ISSN 0022-5142
Volume 51, Number 2
1990

Journal of the Science of Food and Agriculture

Published for the Society of Chemical Industry by Elsevier Applied Science

Journal of the Science of Food and Agriculture publishes original research and critical reviews in agriculture and food science, with particular emphasis on interdisciplinary studies at the agriculture/food interface. The journal covers fundamental and applied research and development of the production of crops and animals, comprising:

- Studies of plant and animal husbandry, physiology, yield and quality
- The interaction of agriculture with forestry and the environment
- Production and processing of human food, fibre, and animal feed
- Nutritional quality and sensory analysis, flavour, texture, safety and toxicity of important human foodstuffs, beverages and alcoholic drinks

The journal also carries reports of scientific meetings of the SCI Food Group, SCI Agriculture Group, and joint meetings with other societies.

JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE

VOL. 51 1990

Published for the SOCIETY OF CHEMICAL INDUSTRY
by ELSEVIER APPLIED SCIENCE

Printed and bound in Great Britain

J Sci Food Agric 1990, **51**, 145–161

Determination of Lignin in Herbaceous Plants by an Improved Acetyl Bromide Procedure

Kenji Iiyama* and Adrian F A Wallis‡

Division of Forestry and Forest Products, CSIRO, Private Bag 10, Clayton, Victoria 3168, Australia

(Received 9 May 1989; revised version received 17 July 1989; accepted 22 August 1989)

ABSTRACT

A modified acetyl bromide procedure is proposed for the spectrophotometric determination of lignin in herbaceous plants. The digestion with 25% acetyl bromide (AcBr) in acetic acid at 70°C is improved by including 4% perchloric acid in the digesting solutions. This allows for more rapid, complete digestion and the use of coarser samples. On the basis of infrared spectra and nitrobenzene oxidation data for milled sample lignins, the value of 20·0 g^{-1} litre cm^{-1} for the specific absorption coefficient of AcBr-treated lignins of herbaceous plant samples gave lignin values which were consistent with nitrobenzene oxidation data, but were higher than those obtained by the acid detergent lignin method and the earlier AcBr method proposed for grasses. The lower lignin values obtained in the latter methods are considered to be due to partial lignin solubilisation in the sulphuric acid digestion.

Key words: Herbaceous plants, grasses, legumes, hardwoods, softwoods, lignin, protein, cinnamic acids, milled sample lignins, lignin determination, acid detergent lignin, acetyl bromide, ultraviolet spectra, nitrobenzene oxidation.

INTRODUCTION

Measurement of the lignin content of herbaceous plant material is generally more difficult than that of woody material, because the former contains proteins and

* Present address: Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.
‡ To whom correspondence should be addressed.

chemically bonded cinnamic acids. Thus application of the usual sulphuric acid method (Browning 1967) to the analysis gives an overestimated value for the lignin content, as proteins co-precipitate with the lignin (Norman and Jenkins 1934). This has led to methods in which the proteins were removed, either by an enzymic pretreatment with pepsin (Crampton and Maynard 1938) or by an acidic detergent treatment (Van Soest 1963) before digestion with the 72% sulphuric acid. In addition, the cinnamic acids are probably rendered soluble in the acidic medium and are estimated as acid-soluble lignin (Lai and Sarkanen 1971), which in turn would lead to inflated values for the total lignin content.

Johnson *et al* (1961) devised a method for determining lignin in wood which is rapid and requires only small samples. In this method, the woodmeal samples are dissolved in a solution of 25% w/w acetyl bromide (AcBr) in acetic acid at 70°C, and the lignin contents are obtained by measurement of the ultraviolet (UV) absorbance of the resulting solutions at 280 nm. The method requires finely ground samples, which are sometimes not completely dissolved after the digestion. We recently proposed an improved AcBr procedure based on adding perchloric acid ($HClO_4$) to the digesting medium, which gave faster and complete dissolution of the samples (Iiyama and Wallis 1988a). This improved method has also been applied to analysing lignin in polyphenol-containing *Eucalyptus* woods (Iiyama and Wallis 1988b).

The AcBr procedure developed for the analysis of lignin in wood samples (Johnson *et al* 1961) was adapted by Morrison for determining lignin in grasses (Morrison 1972a) and legumes (Morrison 1972b). Morrison used the sulphuric acid method (Ellis *et al* 1946), as modified by Waite *et al* (1964), to obtain reference lignin values. The AcBr method was found to be advantageous for both grasses and legumes in that proteins did not give rise to UV absorbance at 280 nm, although the chemically linked cinnamic acid moieties did give absorbance and were measured as lignin. In this report we describe the application of our improved AcBr method to a variety of herbaceous plant samples, and we propose the adoption of an alternative to Morrison's procedure for calculating lignin contents from the UV absorption of the AcBr digests at 280 nm. Use of the new calculation procedure gives lignin values for non-legume samples which are consistent with nitrobenzene oxidation data, but are significantly lower than those obtained by the application of Morrison's method (1972a).

EXPERIMENTAL

Instruments

UV spectra were measured on a Varian Superscan 3 spectrophotometer. Fourier transform infrared (FT–IR) spectra were obtained for KBr discs (1% sample, 64 scans) on an Alpha Centauri FT–IR spectrometer (Mattson Instruments Inc, Madison, Wis, USA). Gas chromatography (GC) was carried out on a Hewlett-Packard 5830A instrument equipped for operation with capillary columns and flame ionisation detection. GC–mass spectrometry (GC–MS) was performed on a Hewlett-Packard 5995A instrument.

Chemicals and materials

The phenol 3-ethoxy-4-hydroxybenzaldehyde, the phenolic acids (E)-*p*-coumaric acid and (E)-ferulic acid, the proteins casein and trypsin, and the amino acid tyrosine, were obtained from commercial sources.

The wheat (*Triticum vulgare* Vill) internode meal was from mature plants donated by Professor B A Stone, La Trobe University. The lucerne (*Medicago sativa* L) was a commercial sample of immature material. The wheat, ryegrass (*Lolium multiflorum* Lam), rice (*Oryza sativa* L), safflower (*Carthamus tinctorius* L), leucaena (*Leucaena leucocephala* (Lam) Wit), sub-clover (*Trifolium subterraneum* L), barley (*Hordeum vulgare* L) and hay samples were a gift from Dr G A Pearce, Melbourne University.

The milled wheat internode and rice internode lignins were prepared by the method of Björkman (1956) from the 80% v/v aqueous ethanol extracted materials. The lignins contained 3% protein (% N × 6·25) and 2·4 and 2·8% w/w carbohydrates respectively (measured as alditol acetates (Blakeney *et al* 1983) after hydrolysis of the lignin samples with 1 M sulphuric acid at 100°C for 2 h). The milled lignins from *Eucalyptus regnans* F Muell and *Pinus radiata* D Don woods were prepared by the method of Bland and Menshun (1970, 1973), and had methoxyl contents of 21·6 and 14·7% w/w, respectively. The exploded sugarcane (*Saccharum officinarum* L) bagasse pulps and lignin were obtained by the methods of Wallis and Wearne (1985).

Preparation of lignocellulose samples for analysis

All samples except the lignins and sugarcane bagasse pulps were ground in a Wiley mill to pass a sieve with 420-μm apertures, and the milled samples (1·0 g) were extracted successively with 100 ml boiling 80% v/v aqueous ethanol for 1·5 h (three times) and with boiling chloroform for 1 h to remove non-cell-wall materials. The extracted samples were dried in a vacuum oven at 60°C and 20 mm pressure.

Acid detergent lignin analyses

These analyses were carried out on the extracted samples by the method of Goering and Van Soest (1970).

Klason lignin and acid soluble lignin determinations

Klason lignin analyses were obtained by Appita standard method P11s-78 (Appita 1978), and involved treating the sample successively with 72% sulphuric acid for 2 h at 20°C and boiling 3% v/v aqueous sulphuric acid for 4 h. Acid soluble lignin was determined by the method of Bland and Menshun (1971). The specific absorption coefficient (SAC) value employed for estimating the acid soluble lignin was 100 g^{-1} litre cm^{-1}.

Lignin analyses by acetyl bromide digestion

Conventional method (Morrison 1972a,b)

The method of Morrison (1972a,b) was followed, except that the digestion was carried out in sealed containers with a tenth the amount of sample to be analysed and half the amount of AcBr reagent.

The sample (4–6 mg for lignocellulose, 1–2 mg for lignins) was placed in a 15-ml glass reaction bottle with a solution of 25% (w/w) AcBr in acetic acid (2·5 ml). The bottle was sealed with a PTFE-coated silicone cap and placed in an oven at 70±0·2°C for 30 min. The bottle was shaken at 10-min intervals to promote dissolution of the sample. After digestion, the mixture was cooled and transferred to a 50-ml volumetric flask containing 2·5 ml 2 M sodium hydroxide and 12 ml acetic acid, and 7·5 M hydroxylamine hydrochloride (0·5 ml) was added to the combined solution. The bottle was rinsed with acetic acid, and the solution was made up to 50 ml with the same solvent. The UV absorption spectrum of the resulting solution was measured against a blank solution which was run in conjunction with the sample. The lignin content of the samples was determined by measuring the absorbances at 280 nm and using the equation of Morrison (1972a) for samples other than legumes:

$$\% \text{ Lignin} = 3{\cdot}37 \times \text{absorbance/sample concn (g litre}^{-1}) - 1{\cdot}05$$

The equation used for the legume samples was:

$$\% \text{ Lignin} = 5{\cdot}12 \times \text{absorbance/sample concn (g litre}^{-1}) - 0{\cdot}74$$

Modified method (Iiyama and Wallis 1988a)

The samples were digested with solutions of 25% (w/w) AcBr in acetic acid as above, except that $HClO_4$ (70%, 0·1 ml) was included in the digesting solution. After 30 min at 70±0·2°C, the reaction bottles were cooled with ice and transferred to a 50-ml volumetric flask containing 2 M sodium hydroxide (10 ml) and acetic acid (12 ml). It was not necessary to add hydroxylamine hydrochloride (cf the above method). The bottle was rinsed, and the solution was made up to 50 ml, with acetic acid. The solution was analysed by UV spectrometry as above. The lignin content of the samples was determined by measuring the absorbances at 280 nm using the SAC value for lignin, 20·0 g^{-1} litre cm^{-1}.

Reaction of proteins and cinnamic acids with acetyl bromide

Samples of the cinnamic acids or proteins (3–5 mg) were digested with 25% (w/w) AcBr in acetic acid solution (2·5 ml) containing 70% $HClO_4$ (0·1 ml), as for the modified method above.

Nitrobenzene oxidation

The method used was a modification of that reported by Garland *et al* (1986). The samples (30–50 mg), 2 M potassium hydroxide (2·5 ml) and nitrobenzene (0·25 ml) were placed in 20-ml stainless steel autoclaves in a rocking hot air bath and kept at 175°C for 2 h. After cooling, the contents were transferred to a beaker and the residual contents of the autoclave were washed into the beaker with a small amount of 0·1 M potassium hydroxide. A solution of 3·0 g litre^{-1} 3-ethoxy-4-hydroxybenzaldehyde in dichloromethane (100 µl) was added as the internal standard. The mixture was extracted with dichloromethane (3 × 30 ml), and the extracts were discarded. The aqueous phase was acidified with 1 M hydrochloric acid to pH 2, and the acidified solution was extracted with dichloromethane

(3 × 25 ml). The combined extracts were washed successively with water (30 ml), dried with sodium sulphate, and evaporated to dryness. The sample was transferred to a septum vial in a small amount of dichloromethane, which was removed with a stream of dry nitrogen. The residue was silylated with N,O-bis(trimethylsilyl)-trifluoroacetamide (100 μl) at 100°C for 1 h. After removal of excess silylating agent under nitrogen, the mixture was dissolved in 200 μl dichloromethane and analysed by GC on a BP1 bonded phase vitreous silica capillary column (12 m × 0·2 mm id) (Scientific Glass Engineering Pty Ltd, Melbourne) in the 'split' injection mode. GC conditions were: injector and detector temperature, 250°C; oven temperature, 150°C for 3 min, then programmed at 10°C min^{-1} to 230°C and 1 min at 230°C; linear flow rate of helium through column, 35 m min^{-1}. The peaks in the chromatograms were identified by GC–MS analysis.

RESULTS AND DISCUSSION

Application of the modified AcBr digestion to herbaceous plant samples

We have found that, for wood samples, digestion with 25% w/w AcBr solutions containing 4% w/w $HClO_4$ at 70°C was faster, and could be applied to coarser samples, than solutions which did not contain $HClO_4$ (Iiyama and Wallis 1988a). In addition, the $HClO_4$-assisted digestions allowed complete dissolution for some samples which were not completely dissolved after digestion for long periods of time with 25% AcBr solutions which did not contain $HClO_4$. A range of herbaceous plant samples gave similar results. When sections of wheat and rice internodes (3–5 mm long) were treated for 30 min at 70°C with the AcBr/$HClO_4$ solution, they were completely dissolved whereas, in the solution that did not contain $HClO_4$, the internode samples remained largely undissolved. The Wiley-milled herbaceous plant samples were not completely dissolved by treatment with the unmodified AcBr solutions, which corroborates the work of Morrison (1972a). However, the $HClO_4$-assisted digestions gave no residue, except for samples containing significant amounts of inorganic constituents, eg rice internodes, which gave residual inorganic material. Although it was ascertained that the undigested material from the unassisted digestion consisted almost entirely of protein (Morrison 1972a), it was still advantageous to effect complete dissolution of the cell wall material so that there was no possibility of a lignin fraction remaining undissolved.

After treatment with the AcBr solution, the digestion mixture was diluted with acetic acid and reacted with hydroxylamine hydrochloride to remove polybromide anions which absorb strongly at 280 nm (Johnson *et al* 1961). In our modified method (Iiyama and Wallis 1988a), we have found that sodium hydroxide solution is effective for the same purpose, and this procedure applied to digests of herbaceous plant samples was also successful. The UV spectra of rice internode meal after digestion with both the $HClO_4$-assisted (modified method) and unmodified AcBr solutions (conventional method) for 30 min at 70°C are given in Fig 1. The modified method gave lower UV SAC values than the conventional method, which contrasts with the earlier work with woodmeals in which similar SAC values were obtained by

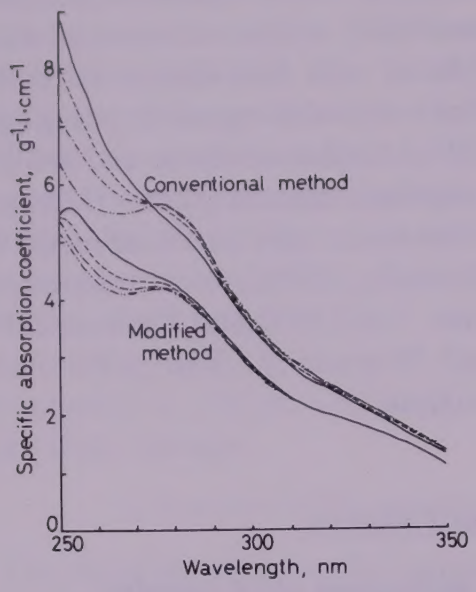


Fig 1. Changes in ultraviolet spectra on standing of diluted AcBr solutions of rice internode meal obtained by the conventional (Morrison 1972a) and modified procedures. ——: Standing time 2 min after dilution; – – –: 30 min; –·–: 60 min; –··–: 24 h.

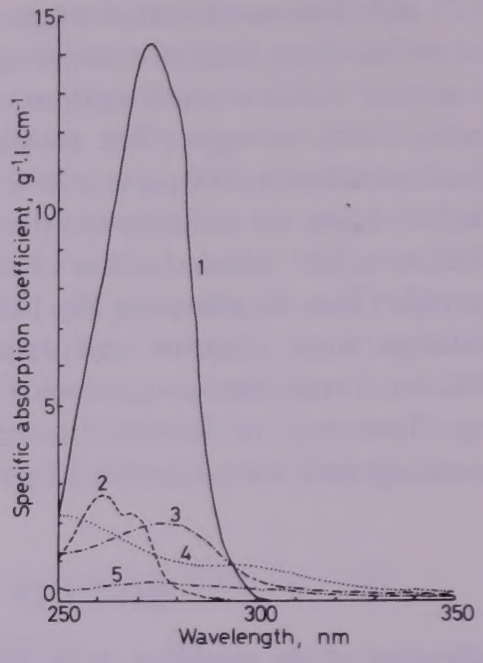


Fig 2. Ultraviolet spectra of tyrosine and proteins in acetic acid solutions before and after the AcBr treatment. ——: Tyrosine (curve 1), – – –: AcBr-treated tyrosine (curve 2); –·–: casein (curve 3); ·····: AcBr-treated casein (curve 4); –··–: AcBr-treated trypsin (curve 5).

both methods (Iiyama and Wallis 1988a). The SAC values at 280 nm of the rice internode digests were more stable for the modified method than for the conventional method; for the former solutions, there was a gradual decrease in SAC values whereas for the latter the SACs increased, and after standing for 30 min this increase was significant. Thus for the reasons given above it is advantageous to use the modified AcBr digestion for herbaceous plant materials.

Morrison (1972a) found that proteins treated with the AcBr solution did not give rise to significant UV absorption at 280 nm. The UV spectra of selected proteins both before and after treatment with the 25% w/w AcBr solution in acetic acid are depicted in Fig 2. Tyrosine, a monomeric constituent of plant proteins, has a *p*-hydroxyphenyl group and is moderately UV absorbing (curve 1). However, after AcBr treatment the UV absorption was drastically lowered, and there was a shift in absorption to lower wavelengths so that the SAC at 280 nm was very low (curve 2). This effect is typical for 4-substituted phenols (Iiyama and Wallis 1989). For casein (curve 3) UV absorption was low, and the SAC at 280 nm was lowered further after AcBr treatment (curve 4). The UV spectrum of AcBr-treated trypsin showed only low absorbances (curve 5). The SAC values of the treated proteins at 280 nm were $\leq 1\ g^{-1}$ litre cm^{-1}, which were low compared with that of lignin (20·0 g^{-1} litre cm^{-1}) (Iiyama and Wallis 1988a). This supports the contention that the contribution of proteins to the absorbance at 280 nm of the AcBr digests is only minor.

Lignins of certain herbaceous plants contain bound cinnamic acid elements which make a contribution to the UV absorption at 280 nm of AcBr digests of the cell wall materials (Morrison 1972a). These cinnamic acids are linked to lignin

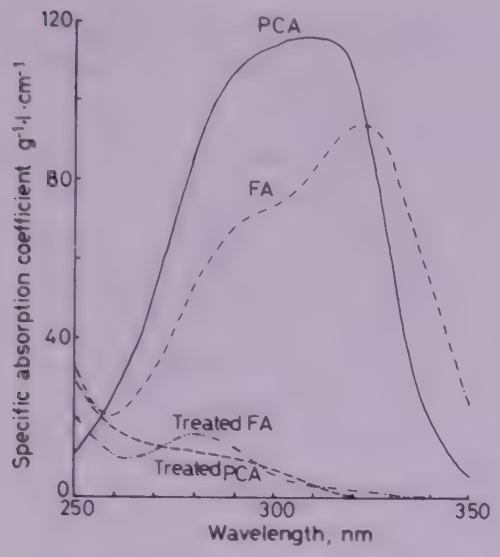


Fig 3. Ultraviolet spectra of phenolic acids in acetic acid solutions before and after AcBr treatment. ——: *p*-Coumaric acid (PCA); –·–: ferulic acid (FA); – – – –: AcBr-treated PCA; –··–: AcBr-treated FA.

through ester bonds (Higuchi *et al* 1967a,b) and through ether bonds (Scalbert *et al* 1985) and to hemicelluloses (Hartley 1973; Mueller-Harvey *et al* 1986). A recent report (Morrison 1988) has suggested the use of a pyridine/pyrrolidine mixture as a pretreatment for barley straw before the AcBr lignin determination to cleave ester linkages. However, the pretreatment would not release the ether-linked cinnamic acids. The effect of AcBr treatment on the UV spectra of *p*-coumaric acid and ferulic acid is shown in Fig 3. The parent acids are very strongly UV absorbing with absorption maxima at 312 nm and 322 nm, respectively, and both have a high SAC at 280 nm. After the AcBr treatment, the absorption maxima were at lower wavelengths (280 nm), and the high absorptions were severely reduced. Morrison (1980) has found that the UV spectra of the AcBr digests of a number of plant samples have significant absorption at wavelengths >300 nm, which he ascribes to the presence of phenolic acids. Our results show that the AcBr-treated phenolic acids do not have high absorbances at wavelengths >300 nm, and these high wavelength absorbances in the AcBr-treated samples are more likely to be due to carbonyl groups conjugated to the aromatic rings (Iiyama and Wallis 1989). The *p*-hydroxycinnamic acids absorb UV light because of their extended chromophoric system through the double bond conjugated to the aromatic ring. This double bond system is evidently destroyed by the AcBr treatment, probably by addition of hydrogen bromide to give saturated structures. The treated *p*-coumaric and ferulic acids gave SAC values at 280 nm of 11·0 and 15·8 g^{-1} litre cm^{-1} respectively, which, although somewhat lower than that of lignin (20·0 g^{-1} litre cm^{-1}), would mean that if present they would make a significant contribution to the lignin content obtained by the AcBr procedure. However, the amount of cinnamic acids in herbaceous plants is generally less than 10% of the amounts of lignin (Higuchi *et al* 1967a,b; Scalbert *et al* 1986), so their presence would not lead to abnormally high lignin values.

Determination of SAC value for AcBr-treated lignins

Calibration curves for the relationship between lignin contents of herbaceous plant samples and SACs of AcBr digests may be generated either by measuring lignin

contents by alternative procedures, or by using isolated lignins as standard samples. Both approaches have their disadvantages; in the former case it is often difficult to obtain reliable lignin contents with alternative methods and in the latter case the isolation of high purity lignins is not an easy task. In the first application of the AcBr method for determining lignin in herbaceous plant samples, Morrison derived relationships between UV SAC values of the AcBr digests at 280 nm and the sulphuric acid lignin content for grasses (1972a) and legumes (1972b). The sulphuric acid method employed for obtaining the lignin contents (Ellis *et al* 1946; Waite *et al* 1964) in Morrison's study probably gave lignin values which were too low, because no account was made of the lignin solubilised by the boiling dilute sulphuric acid pretreatment and the acid-soluble lignin formed during the sulphuric acid digestion. Morrison (1972a) has commented on the problems associated with the determination of lignin by the method of Ellis *et al* (1946).

Although some workers have used Morrison's equations for determining lignin contents from absorbances of AcBr digests at 280 nm, others have used different preparations as lignin reference substances. Brillouet and Riochet (1983) used beech kraft lignin as a reference lignin when determining the AcBr lignin content of lupin hulls. Al-Ani and Smith (1988) based the AcBr lignin content of sugarcane bagasse on ferulic acid as a lignin standard, and Sharma *et al* (1986) used guaiacol as a lignin standard for determining the AcBr lignin content of rice stems and culms. None of these substances has been shown to be suitable as AcBr lignin standards.

We have chosen to use milled sample lignin preparations prepared by the procedure of Björkman (1956) as standards for the lignin in the samples. Scalbert *et al* (1986) have commented on the difficulties in preparing pure milled wheat straw lignin. We prepared milled wheat and rice internode lignins with combined protein and carbohydrate impurities of 5·4 and 6·8 % respectively, and these samples would also contain cinnamic acid residues. Milled wood lignins from a hardwood, *E regnans*, and a softwood, *P radiata*, were also studied for comparative purposes. The FT–IR spectra of the milled sample lignins are presented in Fig 4. The spectra of the graminaceous lignins resemble that of milled wheat straw lignin (Scalbert *et al* 1986) and milled bamboo lignin (Faix and Beinhoff 1988), and compared with the hardwood and softwood lignin spectra the graminaceous lignins have features common to both lignin types. In particular, the band at 1220 cm^{-1} which is

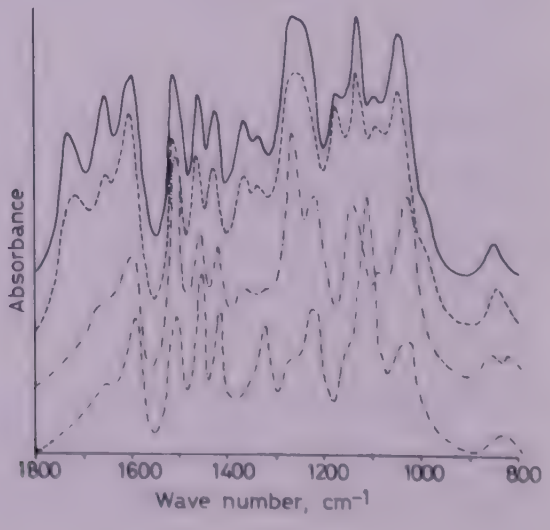


Fig 4. FT–IR spectra of milled sample lignins from various plants ——: Wheat internode, – – – –: rice internode *E regnans*; – · – ·: *P radiata*.

prominent in the hardwood (syringyl–guaiacyl) lignin and that at 1270 cm^{-1}, characteristic of the softwood (guaiacyl) lignin, are of equal prominence in the graminaceous lignin spectra. Thus the graminaceous lignins appear to be guaiacyl–syringyl lignins, with syringyl contents lower than that for the *E regnans* milled wood lignin, which is consistent with the results of Faix and Beinhoff (1988) for bamboo lignin. The bands which appear at 1657 and 1727 cm^{-1} in the IR spectra of the wheat and rice lignins may be assigned to the carbonyl stretching vibrations of proteins and cinnamic acid esters, respectively. We have found that for a wide range of hardwoods and softwoods and derived pulps, use of our modified AcBr method with $HClO_4$ and calculation with an SAC value of 20·0 g^{-1} litre cm^{-1} gave lignin values which were in close agreement with those obtained by the sulphuric acid procedure (Iiyama and Wallis 1988a,b). Because there was no reason to expect the SAC of the treated lignins from herbaceous plants to be different from that of the wood lignins (Iiyama and Wallis 1989), we thus chose the same SAC value for determining lignin in the AcBr-treated herbaceous plant samples.

Results from application of the conventional AcBr method (Morrison 1972a) and the modified method to the analysis of lignin in the milled sample lignin preparations and to wheat internode meal and *E regnans* woodmeal are given in Table 1. The lignin contents are given as percentages of unextracted samples. For the lignins, the modified AcBr method gave analytical values in the range 90–100%, whereas the conventional method for grasses (Morrison 1972a) produced values from 60 to 67%. The modified method is thus more appropriate for these samples. Nitrobenzene oxidations were carried out on the four milled sample lignins, and the molar ratio of the products with syringyl nuclei, syringaldehyde and syringic acid to the products with guaiacyl nuclei, vanillin and vanillic acid (S/V ratio) showed that the graminaceous lignins had a syringyl content intermediate between that of the hardwood and softwood lignins, which is consistent with earlier reports (Creighton *et al* 1944; Higuchi *et al* 1967b; Kondo *et al* 1986; Scalbert *et al* 1986). The lower total aldehyde yield and S/V ratio from the milled internode lignin from rice compared with that of wheat is probably due to the lower maturity of the former sample.

The yields of aldehydes obtained based on the percentage of the lignin after nitrobenzene oxidation of various lignocellulosic samples are typically 35–51% for hardwoods and 15–24% for softwoods (Creighton *et al* 1944) whereas for herbaceous plants the yields are in the range 8–33% (Higuchi *et al* 1967a; Kondo *et al* 1986). Thus the application of our modified method to the analysis of lignin in an *E regnans* woodmeal and a wheat straw meal gave yield values for the aldehydes from the nitrobenzene oxidations which were within the expected range (Table 1), whereas those calculated on the basis of the AcBr lignin content obtained by Morrison's procedure were considerably higher than expected. This gives further credence to the validity of the modified method and the SAC value for the AcBr-treated graminaceous lignins.

Lignin analyses of herbaceous plant samples

A range of herbaceous plant samples were analysed for lignin by both the conventional and modified AcBr procedures, and by the acid detergent method of

TABLE 1
Lignin analyses and nitrobenzene oxidations[a]

Sample	*AcBr lignin*		*S/V*[d]	*Nitrobenzene oxidation*		
				Total aldehyde, % based on:		
	Conventional method[b]	*Modified method*[c]		*ODM*[e]	*AcBr lignin*	
					Conventional method[b]	*Modified method*[c]
Milled wheat internode lignin	66·5	100·2	1·1	25·2	38·0	25·1
Milled rice internode lignin	62·6	94·4	0·6	11·7	18·7	12·4
Milled *Eucalyptus regnans* lignin	59·6	90·0	3·0	19·8	33·2	22·0
Milled *Pinus radiata* lignin	62·3	94·0	0·0	18·5	29·7	19·7
Wheat internode meal	6·5	11·0	1·0	3·0	46·9	22·7
E regnans woodmeal	14·9	23·6	4·5	9·7	65·1	41·3

[a] Based on original (unextracted) dry materials.
[b] Determined according to Morrison (1972a).
[c] This work, specific absorption coefficient for lignin 20·0 g^{-1} litre cm^{-1}.
[d] Molar ratio of syringyl to guaiacyl nuclei.
[e] Oven dry matter.

Goering and Van Soest (1970), and they were also oxidised with alkaline nitrobenzene (Table 2). In addition, some samples derived from sugarcane bagasse were analysed by the AcBr procedures and by the sulphuric acid (Klason) method. The amounts of samples taken for the AcBr digestions were a tenth of those used by Morrison (1972a,b). Use of the smaller sample sizes allowed the use of less reagents, although the precision of the analyses was still good; repeated analyses gave a coefficient of variation value of 3·0%. The samples listed in Table 2 were extracted successively with 80% aqueous ethanol and chloroform before they were analysed for lignin. There was significantly more material extracted from the immature than from the mature samples. The lignin contents of the samples other than the legumes as determined by the various procedures generally decreased in the order modified AcBr method > conventional AcBr method > acid detergent method. However, the legume samples analysed for lignin by the conventional method in which a different equation was used (Morrison 1972b) gave similar values to those obtained by the modified method (Table 2). The lignin contents of the mature samples were higher than those of immature samples, which is reflected in the UV spectra of the digests from the AcBr treatments (Figs 5–7). All spectra have absorption maxima or shoulders at 280 nm except the barley grain, which does not contain lignin. Morrison (1980) found significant UV absorption at wavelengths >300 nm for some AcBr-treated herbaceous samples, and in some of our spectra, notably those in Fig 7, we have observed a similar effect. For the samples derived from sugarcane bagasse, the total lignin values obtained by the sulphuric acid (Klason) method were similar to those of the modified AcBr method, but higher than those obtained by the conventional AcBr method (Table 3).

Nitrobenzene oxidation of the samples in Table 2 except the legumes showed that the total aldehyde yields were within the range expected for herbaceous plants based on the lignin contents obtained by the modified AcBr method, but were higher than expected when based on lignin contents obtained by the conventional AcBr method or the acid detergent method. The yields of the total oxidation products from the legume samples based on the lignin contents determined by both AcBr procedures were within the accepted range (Table 2). This indicates that both the acid detergent and conventional AcBr methods give erroneously low values for lignin contents of herbaceous samples other than legumes, and that the conventional AcBr procedure applied to legumes gives a correct estimation of the lignin contents. The nitrobenzene oxidation data were consistent with the lignin contents determined by the modified AcBr method. The yields of oxidation products and the S/V ratios were lower for the immature samples than for the mature samples, which is consistent with the observations of Terashima *et al* (1986) who found that at the early stages of lignin formation highly condensed guaiacyl lignin is deposited.

It is well recognised that the acid detergent method for lignin gives an underestimation of the true lignin content (Porter and Singleton 1971; Kondo *et al* 1986, 1987). This is because the lignin is partially solubilised and rendered colloidal by the acid detergent pretreatment before the sulphuric acid digestion. In the sulphuric acid method of Ellis *et al* (1946) for determining lignin, which was used by Morrison (1972a,b) for correlating the lignin contents with UV data on the AcBr digests, the samples are pretreated with an acidic pepsin solution followed by

TABLE 2
Lignin analyses and nitrobenzene oxidations of herbaceous plant samples[a]

Sample	*80% ethanol extracted residue, %*	*AD lignin[b] %*	*AcBr lignin*		*Alkaline nitrobenzene oxidation*				
			Conv[c] %	*Modif[d] %*	*S/V[e]*	*Total yield, % based on:*			
						ODM[f] %	*AcBr lignin*		*AD lignin %*
							Conv %	*Modif %*	
Wheat internode									
Immature	56·2	5·3	5·6	9·2	1·0	2·5	44·2	26·9	46·7
Mature	83·3	11·2	10·4	16·7	1·1	4·7	45·0	28·2	41·8
Wheat sheath									
Immature	67·3	3·3	5·6	9·4	—	—	—	—	—
Mature	83·5	4·9	7·5	12·4	—	—	—	—	—
Wheat blade									
Immature	66·0	2·2	3·4	6·1	0·5	0·6	17·6	9·8	27·3
Mature	75·9	4·1	4·0	7·1	0·5	0·9	23·0	13·0	22·5
Ryegrass internode									
Immature	70·2	—	10·3	16·4	1·3	3·2	31·1	19·5	—
Mature	80·2	6·0	10·3	16·5	1·5	3·3	32·3	20·2	48·2

Ryegrass sheath									
Immature	80·7	—	5·4	9·3	—	—	—	—	—
Mature	82·0	5·2	6·5	10·9	1·1	2·5	38·1	22·7	47·7
Ryegrass blade									
Immature	58·5	1·0	4·4	7·4	0·4	0·5	10·7	6·4	47·0
Mature	77·5	4·5	5·4	9·2	0·5	1·0	18·4	10·8	22·1
Rice straw 1	77·0	4·0	4·3	8·5	1·3	2·9	66·5	33·6	71·5
Rice straw 2	82·7	—	5·3	9·4	1·1	2·8	52·7	29·7	—
Hay	76·0	5·1	5·6	9·5	0·8	1·9	33·6	19·8	36·9
Safflower	78·5	—	4·6	8·0	0·7	1·4	30·1	17·3	10·0
Legumes									
Subclover									
Immature	63·6	—	3·3	3·3	0·4	0·7	22·7	22·7	—
Mature	78·8	—	4·5	4·8	0·5	1·5	33·4	31·3	—
Lucerne	45·4	—	1·2	1·0	0·1	0·1	11·0	13·2	—
Leucaena	66·9	9·7	10·0	9·9	0·9	2·0	19·9	20·1	20·5

[a] Based on original (unextracted) dry materials.
[b] Acid detergent lignin determined according to Goering and Van Soest (1970).
[c] Determined by Morrison's procedures for grasses (1972*a*) and legumes (1972*b*).
[d] This work, specific absorption coefficient for lignin 20·0 g^{-1} litre cm^{-1}.
[e] Molar ratio of syringyl to guaiacyl nuclei.
[f] Oven dry matter.

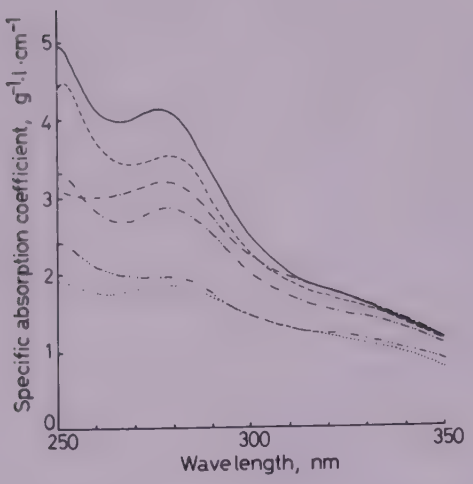


Fig 5. UV spectra of AcBr-treated wheat straw components. – – – –: Internode (immature); ——: internode (mature); –··–: sheath (immature); –·–: sheath (mature); –···–: blade (immature); ·····: blade (mature).

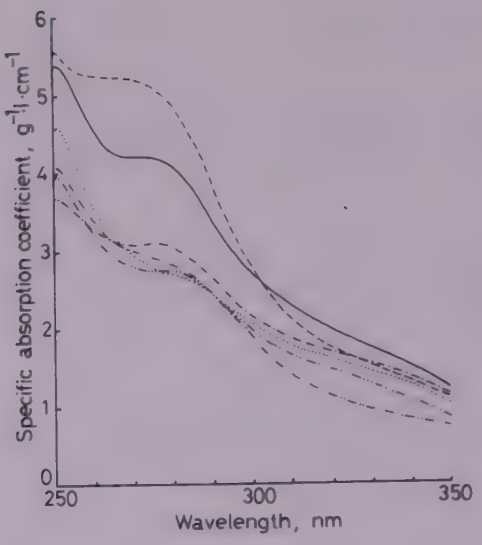


Fig 6. UV spectra of AcBr-treated ryegrass components. – – – –: Internode (immature); ——: internode (mature); –··–: sheath (immature); –·–: sheath (mature); –···–: blade (immature); ·····: blade (mature).

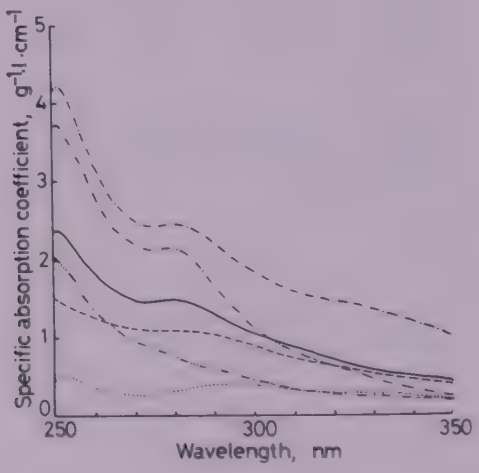


Fig 7. UV spectra of AcBr-treated herbaceous plants. ——: Subclover (immature); – – –: subclover (mature); – – –: hay; –···–: safflower; –···–: lucerne; ·····: barley grain.

TABLE 3

Lignin analyses of pulps and lignin from sugarcane bagasse

Sample	*H_2SO_4 lignin*			*AcBr lignin*	
	KL[a] %	*AS*[b] %	*Total* %	*Conventional*[c] %	*Modified*[d] %
Exploded bagasse pulp	29·0	2·4	31·4	20·1	31·4
Alkali-extracted exploded pulp	10·2	0·5	10·7	5·0	9·0
Exploded pulp lignin	—	—	—	69·0	104·2

[a] Klason lignin.
[b] Acid-soluble lignin.
[c] Determined according to Morrison (1972a).
[d] This work. Specific absorption coefficient for lignin 20·0 g^{-1} litre cm^{-1}.

boiling dilute sulphuric acid. Kondo *et al* (1987) have shown that the lignins of herbaceous plants are partially solubilised after treatment with boiling 0·5 M sulphuric acid for 1 h, and the lignins of grasses are solubilised to a greater extent than those of legumes. A part of the lignin would also be expected as an acid-soluble fraction after digestion with the 72% sulphuric acid. The differential solubilities in acid of the lignin in legumes and in other herbaceous plant materials could account for the apparent differences in the correlations between UV data of AcBr digests and lignin contents. Both of Morrison's correlations do not show a direct proportionality between the UV SAC at 280 nm and lignin content. This could be due to a dependence of lignin content either on the structure of the lignins of herbaceous plant samples or on the amounts of lignin extracted with acid. In support of the latter proposition, Kondo *et al* (1987) found that a greater proportion of lignin is removed from immature herbaceous plant samples than from mature samples. We thus recommend the adoption of our modified method for digestion of herbaceous plant samples with AcBr solution, and the use of the UV SAC value of 20·0 g^{-1} litre cm^{-1} for lignin for the calculation of the lignin content.

ACKNOWLEDGEMENTS

The authors are grateful to Professor B A Stone, La Trobe University, Melbourne, for the sample of wheat internode meal, and to Dr G A Pearce, School of Agriculture, Melbourne University, for many of the samples and for measuring the acid detergent lignin values.

REFERENCES

Al-Ani F, Smith J E 1988 Effect of chemical pretreatments on the fermentation and ultimate digestibility of bagasse by *Phanerochaete chrysosporium*. *J Sci Food Agric* **42** 19–28.

Appita 1978 Appita standard method P11s-78. Klason lignin in wood and pulp. Technical Association of the Australian and New Zealand Pulp and Paper Industry Inc, Victoria.

Björkman A 1956 Studies on finely divided wood. Part 1. Extraction of lignin with neutral solvents. *Sven Papperstidn* **59** 477–485.

Blakeney A B, Harris P J, Henry R J, Stone B A 1983 A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr Res* **113** 291–299.

Bland D E, Menshun M 1970 The composition and reactive groups of the lignin of *Eucalyptus regnans*. *Appita* **23** 427–433.

Bland D E, Menshun M 1971 Determination of total lignin and polyphenol in eucalypt woods. *Appita* **25** 110–115.

Bland D E, Menshun M 1973 The effect of milling and alkali treatments on the accessibility of the lignins of *Pinus radiata* and *Eucalyptus regnans*. *Holzforschung* **27** 33–35.

Brillouet J-M, Riochet D 1983 Cell wall polysaccharides and lignin in cotyledons and hulls of seeds from various lupin (*Lupinus* L.) species. *J Sci Food Agric* **34** 861–868.

Browning B L 1967 *Methods of Wood Chemistry*, Vol. II. Interscience Publishers, New York, pp 785–793.

Crampton E W, Maynard L A 1938 The relation of cellulose and lignin content to animal feeds. *J Nutr* **15** 383–395.

Creighton R H J, Gibbs R D, Hibbert H 1944 Lignin and related compounds. LXXV. Alkaline nitrobenzene oxidation of plant materials and application to taxonomic classification. *J Amer Chem Soc* **66** 32–37.

Ellis G H, Matrone G, Maynard L A 1946 A 72 percent H_2SO_4 method for the determination of lignin and its use in animal nutrition studies. *J Anim Sci* **5** 285–297.

Faix O, Beinhoff O 1988 FTIR spectra of milled wood lignins and lignin polymer models (DHP's) with enhanced resolution obtained by deconvolution. *J Wood Chem Technol* **8** 505–522.

Garland C P, James F C, Nelson P J, Wallis A F A 1986 Chemical analyses and oxidative studies of *Eucalyptus regnans*, *E. diversicolor*, *E. marginata* and *E. tetrodonta* wood samples. *Appita* **39** 361–368.

Goering H K, Van Soest P J 1970 Forage fiber analyses (apparatus, reagents, procedures and some applications). *Agricultural Handbook No. 379*, Agricultural Research Service, United States Department of Agriculture, US Government Printing Office, Washington, DC, pp 1–20.

Hartley R D 1973 Carbohydrate esters of ferulic acid as components of cell walls of *Lolium multiflorum*. *Phytochem* **12** 661–665.

Higuchi T, Ito Y, Kawamura I 1967a *p*-Hydroxyphenylpropane component of grass lignin and rôle of tyrosine-ammonia lyase in its formation. *Phytochem* **6** 875–881.

Higuchi T, Ito Y, Shimada N, Kawamura I 1967b Chemical properties of milled wood lignin of grasses. *Phytochem* **6** 1551–1556.

Iiyama K, Wallis A F A 1988a An improved acetyl bromide procedure for determining lignin in woods and wood pulps. *Wood Sci Technol* **22** 271–280.

Iiyama K, Wallis A F A 1988b Spectrophotometric determination of lignin in polyphenol-containing *Eucalyptus* woods by the acetyl bromide method. *Appita* **41** 442–446.

Iiyama K, Wallis A F A 1989 Effect of acetyl bromide treatment on the ultraviolet spectra of lignin model compounds. *Holzforschung* **43** 309–316.

Johnson D B, Moore W E, Zank L C 1961 The spectrophotometric determination of lignin in small wood samples. *Tappi* **44** 793–798.

Kondo T, Mizuno K, Kato T 1986 Chemical and physical properties of forage plant lignin. I. Differential effect of acid detergent on grass and legume lignin. *Bull Nat Grassl Res Inst* **35** 55–61.

Kondo T, Mizuno K, Kato T 1987 Variation in solubilities of lignin in acid detergent and in alkali. *Nippon Sochi Gakkaishi* **33** 296–299.

Lai Y Z, Sarkanen K V 1971 Isolation and structural studies. In: *Lignins. Occurrence, Formation, Structure and Reactions*, eds Sarkanen K V & Ludwig C H. Wiley-Interscience, New York, p 194.

Morrison I M 1972a A semimicro method for the determination of lignin and its use in predicting the digestibility of forage crops. *J Sci Food Agric* **23** 455–463.

Morrison I M 1972b Improvements in the acetyl bromide technique to determine lignin and digestibility and its application to legumes. *J Sci Food Agric* **23** 1463–1469.

Morrison I M 1980 Hemicellulosic contamination of acid detergent residues and their replacement by cellulose residues in cell wall analysis. *J Sci Food Agric* **31** 639–645.

Morrison I M 1988 Influence of chemical and biological pretreatments on the degradation of lignocellulosic material by biological systems. *J Sci Food Agric* **42** 305–314.

Mueller-Harvey I, Hartley R D, Harris P J, Curzon E H 1986 Linkage of *p*-coumaroyl and feruloyl groups to cell wall polysaccharides of barley straw. *Carbohydr Res* **148** 71–85.

Norman A G, Jenkins S H 1934 The determination of lignin. II. Errors introduced by the presence of proteins. *Biochem J* **28** 2160–2168.

Porter P, Singleton A G 1971 The degradation of lignin and quantitative aspects of ruminant digestion. *Br J Nutr* **25** 3–14.

Scalbert A, Monties B, Lallemand J-Y, Guittet E, Rolando C 1985 Ether linkage between phenolic acids and lignin fractions from wheat straw. *Phytochem* **24** 1359–1362.

Scalbert A, Monties B, Guittet E, Lallemand J Y 1986 Comparison of wheat straw preparations I. Chemical and spectroscopic characterizations. *Holzforschung* **40** 119–127.

Sharma U, Brillouet J-M, Scalbert A, Monties B 1986 Studies on a brittle stem mutant of rice, *Oryza sativa* L.; characterization of lignin fractions, associated phenolic acids and polysaccharides from rice stem. *Agronomie* **6** 265–271.

Terashima N, Fukushima K, Takabe K 1986 Heterogeneity in formation of lignin. VIII. An autoradiographic study on the formation of guaiacyl and syringyl lignin in *Magnolia kobus* DC. *Holzforschung* **40** Suppl 101–105.

Van Soest P J 1963 Use of detergents in analysis of fibrous feeds. II. A rapid method for the determination of fiber and lignin. *J Assoc Offic Agric Chem* **46** 829–835.

Waite R, Johnston M J, Armstrong D G 1964 The evaluation of artificially dried grass as a source of energy for sheep. I. The effect of stage of maturity on the apparent digestibility of rye-grass, cocksfoot and timothy. *J Agric Sci Camb* **62** 391–398.

Wallis A F A, Wearne R H 1985 Fractionation of the polymeric components of hardwoods by autohydrolysis–explosion–extraction. *Appita* **38** 432–437.

J Sci Food Agric 1990, **51**, 163–170

Monomeric and Dimeric Phenolic Constituents of Plant Cell Walls—Possible Factors Influencing Wall Biodegradability

Fatima Eraso* and Roy D Hartley‡

AFRC Institute for Grassland and Animal Production, Maidenhead, Berkshire SL6 5LR, UK

(Received 2 June 1989; accepted 22 August 1989)

ABSTRACT

A range of plant cell walls from graminaceous and leguminous plants were examined qualitatively and quantitatively for monomeric and dimeric phenolic constituents that were released by treatment with sodium hydroxide. The total amounts of phenolics released from the walls of the graminaceous plants varied from 8 to 28 mg g^{-1} *walls compared with less than 3 mg* g^{-1} *walls from the legumes.* p-*Coumaric and ferulic acids were the major components of the monomeric fraction. The cell walls also contained substituted cyclobutanes having molecular weights equal to two* p-*coumaric acid molecules, two ferulic acid molecules or one* p-*coumaric plus one ferulic acid molecule. All the walls contained dehydrodiferulic acid. If it is assumed that the substituted cyclobutanes and dehydrodiferulic acid arise from dimerisation of feruloyl and* p-*coumaroyl groups linked to cell wall polysaccharides, then, for the graminaceous walls, it is calculated that between 5 and 14% of these groups had converted to dimers. This dimerisation process may limit the biodegradability of the wall polysaccharides.*

Key words: Italian ryegrass, barley straw, maize, sorghum, lucerne, red clover, plant cell walls, phenolic constituents, phenolic monomers, phenolic dimers, 4,4′-dihydroxy-α-truxillic acid, dehydrodiferulic acid.

* Present address: Junta de Andalucia, Consejeria de Agricultura, y Pesca, 14071-Cordoba, Spain.

‡ To whom all correspondence should be addressed at: Richard B Russell Agricultural Research Center, Agricultural Research Service, US Department of Agriculture, Athens, Georgia 30613, USA.

INTRODUCTION

There is an increasing interest in the aromatic constituents (lignin and other phenolics) of the plant cell wall as they limit the biodegradability of the wall polysaccharides. This is of importance in animal nutrition as decrease in the digestibility of forage leads to lower rates of animal production (for reviews see Akin 1989; Hartley and Ford 1989). Phenolic constituents of plant cell walls may also be of importance as anti-microbial compounds in relation to disease resistance in plants. It is well established that graminaceous cell walls contain monomeric phenolic constituents, mainly the *trans* isomers of *p*-coumaric (PCA) and ferulic (FA) acids, some of which are ester-linked to arabinoxylans (El-basyouni *et al* 1964; Kuc and Nelson 1964; Hartley 1972; Kato *et al* 1983; Kato and Nevins 1985; Mueller-Harvey *et al* 1986). Other work has shown that small amounts of dehydrodiferulic ('diferulic') acid are also constituents of graminaceous cell walls (Hartley and Jones 1976; Markwalder and Neukom 1976). Recently a substituted cyclobutane, 4,4′-dihydroxy-α-truxillic acid, a photodimer of *p*-coumaric acid, has been reported as a constituent of ryegrass and tropical grasses (Hartley *et al* 1988; Ford and Hartley 1989a,b). These studies have shown that other stereoisomers of this photodimer (ie 'PCA–PCA' type dimers) are present in the walls together with analogous ferulic acid–ferulic acid ('FA–FA') dimers and mixed *p*-coumaric acid–ferulic acid ('PCA–FA') dimers. Theoretically each of these dimer types has 12 stereoisomers (Khan 1966).

As little is known about the amounts of dimers associated with cell walls from temperate and sub-tropical species, the present work examines, by gas chromatography, the major monomeric and dimeric phenolic constituents of economically important graminaceous and leguminous plants.

EXPERIMENTAL

All monomeric compounds were obtained from Koch Light (Haverhill, Suffolk, UK), with the exceptions of *cis*-*p*-coumaric acid (from Aldrich, Milwaukee, WI, USA) and *cis*-ferulic acid which was prepared by exposure of a solution of the *trans* isomer to ultraviolet radiation (Hartley and Jones 1975).

trans,*trans*-Dehydrodiferulic ('diferulic') acid was synthesised as described earlier: the *cis*,*trans* isomer was obtained by exposure of a solution of the *trans*,*trans* isomer to UV radiation (Hartley and Jones 1976). 4,4′-Dihydroxy truxillic acid was synthesised as described earlier (Hartley *et al* 1988); the acid has been shown to be the α isomer by X-ray diffraction and nuclear magnetic resonance studies (Cohen *et al* 1964; Schmidt 1964; Hartley R D, Himmelsbach D S unpublished).

Diethyl ether was redistilled just before use. All solutions of phenolic compounds were manipulated in white fluorescent light to avoid effects of UV radiation including isomerisation (Kahnt 1967; Hartley and Jones 1975).

Plant materials

The following plant materials were evaluated (see appropriate reference for details of growth and harvesting): vegetative shoots of Italian ryegrass (*Lolium multiflorum*

Lam, cv RVP) (Hartley and Jones 1976); barley straw (*Hordeum vulgare* L, cv Julia) (Hartley and Jones 1978a); lucerne mature stem (*Medicago sativa* L, cv Maris Phoenix) (Hartley 1983); stem (base) of red clover (*Trifolium pratense* L, cv Hungaropoly) harvested 2 July (Hartley and Jones 1977); leaf blade, leaf sheath and stem of the brown midrib mutant (bm_3) of maize (*Zea mays* L, cv Troyer Reid) (Hartley and Jones 1978b); and leaf blade plus sheath and mature stem of sorghum (*Sorghum bicolor* L, cv Melkamash 79) (Reed J D, Hoefs S unpublished).

Isolation of plant cell walls

Cell walls were separated by a modification (Hartley *et al* 1974) of the neutral detergent procedure of Van Soest and Wine (1967): sodium sulphite and dekalin were excluded from the detergent solution and the isolated walls were washed with water, followed by acetone and ether then dried over silica gel.

Treatment of cell walls with NaOH

Cell walls (100 ml) were shaken with sodium hydroxide (1 M, 5 ml) at 20°C for 20 h under nitrogen (containing less than 5 mg kg^{-1} oxygen) and filtered (No 1 porosity glass sinter), and the residue was washed with water. Freshly prepared *trans*-sinapic acid (1 g $litre^{-1}$ 1 M NaOH, 0·5 ml) was added to the filtrate plus washings as an internal standard and the solution was acidified to pH 2·5 with 6 M HCl and extracted with freshly distilled diethyl ether (3 × 30 ml). The combined ether extracts were dried over anhydrous sodium sulphate and the solvent was evaporated in a stream of nitrogen. The residue was dried over silica gel and silylated by addition of a mixture of N,O-*bis*(trimethylsily)trifluoroacetamide plus trimethylchlorosilane plus pyridine (1:0·1:1 by volume, 100 μl) and shaken intermittently at 37°C for 20 h.

Gas chromatography

Trimethylsilyl derivatives were separated on a WCOT capillary column coated with a bonded phase CP-SIL 5CB (10 m × 0·22 mm id) and detected by flame ionisation as described earlier (Ford and Hartley 1989b). *trans*-Sinapic acid was used as an internal standard as it was absent from the saponification products from the cell walls. Quantification was performed with a Compaq Deskpro computer (Chrompack-Packard).

A solution of reference *trans*-*p*-coumaric acid, *cis*-*p*-coumaric acid, *trans*-ferulic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-hydroxybenzaldehyde, vanillin, syringaldehyde, *trans*,*trans*-dehydrodiferulic acid, 4,4′-dihydroxy-α-truxillic acid and *trans*-sinapic acid (1 mg of each in 5 ml 1 M NaOH) was acidifed, then extracted with ether, the solvent was removed, and the residue was silylated as described in the preceding section. Each reference compound gave one peak indicating that the fully silylated derivative was obtained under the conditions used. Recoveries of these reference compounds were determined by comparison with direct chromatography of their silyl derivatives and ranged from 85 to 91%. For the quantitative determination of these compounds in plant extracts, results were calculated by taking account of the percentage recovered together with the appropriate response factor. The amounts in the plant extracts of compounds for which no references

TABLE 1

Monomeric phenolic constituents of cell walls of Italian ryegrass shoot, barley straw, lucerne and red clover stem released by sodium hydroxide treatment (mg g^{-1} dry cell walls)

Monomer	*Relative retention time*[a]	*Italian ryegrass shoot*	*Barley straw*	*Lucerne stem*	*Red clover stem*	*SE*
p-Coumaric acid (PCA)						
trans	0·550	0·78	3·54	1·64	0·52	0·095
cis	0·366	0·17	0·30	0·10	0·10	0·036
Ferulic acid (FA)						
trans	0·777	6·65	3·53	0·50	0·29	0·119
cis	0·537	0·47	0·17	0·10	0·10	0·095
p-Hydroxybenzoic acid	0·223	0·10	0·10	0·10	0·10	
Vanillic acid	0·343	0·10	0·15	0·10	0·10	
p-Hydroxybenzaldehyde	0·097	0·10	0·10	0·10	0·10	
Vanillin	0·154	0·11	0·12	0·10	0·10	
Syringaldehyde	0·261	0·10	0·16	0·10	0·10	
Total monomers		8·98	8·17	2·84	1·51	
Ratio PCA:FA[b]		0·13	1·04	2·90	1·59	

SEs are for means of duplicate samples.

[a] By gas chromatography of the fully silylated (trimethylsilyl) derivative (relative to the *trans*-sinapic acid derivative = 1·000).

[b] *Cis* + *trans* isomers.

TABLE 2

Monomeric phenolic constituents of cell walls of maize and sorghum released by sodium hydroxide treatment (mg g^{-1} dry cell walls)

Monomer	*Maize*			*Sorghum*		*SE*
	Leaf blade	*Leaf sheath*	*Stem*	*Leaf blade + sheath*	*Stem*	
p-Coumaric acid (PCA)						
trans	2·25	7·37	19·60	9·26	13·85	0·066
cis	0·19	0·50	0·67	0·47	0·57	0·052
Ferulic acid (FA)						
trans	4·42	9·94	6·88	4·10	4·21	0·041
cis	0·40	0·74	0·54	0·18	0·16	0·091
p-Hydroxybenzoic acid	—[a]	—	0·10	0·10	0·10	
Vanillic acid	0·51	0·10	0·10	0·10	0·10	0·056
p-Hydroxybenzaldehyde	0·11	0·14	0·21	0·10	0·10	0·211
Vanillin	0·10	0·19	0·19	0·10	0·15	0·142
Syringaldehyde	0·10	0·14	0·14	0·15	0·10	0·116
Total monomers	8·08	19·12	28·43	14·56	19·24	
Ratio PCA:FA[b]	0·51	0·74	2·73	2·27	3·28	

SEs are for means of duplicate samples.

[a] Not detectable.

[b] *Cis* + *trans* isomers.

were available were calculated assuming a recovery of 100% and a response factor of 1 with respect to *trans*-sinapic acid.

RESULTS AND DISCUSSION

The amounts of monomeric phenolic constituents found in the cell walls are summarised in Tables 1 and 2. As expected, the graminaceous walls, compared with the legumes, had much higher total contents of phenolic monomers, ranging from 8 to 28 mg g^{-1} cell walls (legumes less than 3 mg g^{-1} cell walls). There was considerable variation in the proportions of compounds that comprised this phenolic fraction but *p*-coumaric and ferulic acids were the major constituents. The stems of maize and sorghum had large amounts of *trans*-*p*-coumaric acid in their walls (maize 20 mg g^{-1} and sorghum 14 mg g^{-1} walls) in agreement with earlier work (Hartley and Jones 1978b; Cherney *et al* 1988); in contrast, leaf blade of maize had only 2 mg of the acid g^{-1} walls. The ratio of *p*-coumaric to ferulic acid was lowest in Italian ryegrass shoots.

TABLE 3

Dimeric phenolic constituents of cell walls of ryegrass, barley straw, lucerne and red clover released by sodium hydroxide treatment (mg g^{-1} dry cell walls)

Dimer	*Relative retention time*[a]	*Italian ryegrass shoot*	*Barley straw*	*Lucerne stem*	*Red clover stem*	*SE*
Dehydrodiferulic acid						
trans,trans	2·135	0·18	0·35	0·10	0·10	3·334
cis,trans	1·940	0·10	0·10	—[b]	—	
Substituted cyclobutane dimers						
4,4′-dihydroxy-α-truxillic acid (PCA–PCA)	1·799	0·11	0·10	0·10	0·10	0·395
PCA–FA (Peak '6')[c]	1·844	0·21	—	—	—	0·143
FA–FA (Peak '8')[c]	1·897	0·56	—	—	—	0·018
FA–ConAlc (Peak '10')[c]	1·939	—	—	0·10	—	
SE						
Total dimers		1·10	0·55	0·30	0·20	
Ratio total dimers:total monomers		0·12	0·07	0·11	0·13	
Unknown compound A	1·309	0·20	0·17	0·10	0·21	0·340
Unknown compound B	1·666	0·21	0·18	—	—	0·285
Unknown compound C	1·785	—	—	—	—	

SEs are for means of duplicate samples.

[a] By gas chromatography of the fully silylated (trimethyl silyl) derivative (relative to the *trans*-sinapic acid derivative = 1·000).

[b] Not detectable.

[c] Derived from *p*-coumaric plus ferulic acid (PCA–FA), ferulic acid (FA–FA) or ferulic acid plus coniferyl alcohol (FA–ConAlc): peak numbers as designated by Ford and Hartley (1989b).

TABLE 4

Dimeric phenolic constituents of cell walls of maize, and sorghum released by sodium hydroxide treatment (mg g^{-1} dry cell walls)

Dimer	*Maize*			*Sorghum*		*SE*
	Leaf blade	*Leaf sheath*	*Stem*	*Leaf blade + sheath*	*Stem*	
Dehydrodiferulic acid						
trans,trans	0·33	1·36	0·92	1·14	0·44	0·273
cis,trans	0·19	0·55	0·57	0·50	0·29	0·173
Substituted cyclobutane dimers						
4,4′-dihydroxy-α-truxillic acid (PCA–PCA)	0·28	0·27	0·10	0·24	0·10	0·373
PCA–FA (Peak '6')[a]	0·20	0·15	0·17	0·18	0·10	0·426
FA–FA (Peak '8')[a]	—[b]	—	—	0·29	0·10	0·078
FA–ConAlc (Peak '10')[a]	—	0·55	0·25	0·31	0·25	0·115
Total dimers	1·00	2·88	2·01	2·66	1·28	
Ratio total dimers: total monomers	0·12	0·15	0·07	0·18	0·07	
Unknown compound A[c]	—	—	—	0·10	0·10	
Unknown compound B[c]	0·14	—	—	0·11	0·10	
Unknown compound C[c]	0·15	0·19	—	0·21	0·10	

SEs are for means of duplicate samples.
[a] See Table 3, note *c*.
[b] Not detectable.
[c] See Table 3.

The contents in the cell walls of dehydrodiferulic acid and substituted cyclobutane dimers (PCA–PCA, PCA–FA and FA–FA types) are listed in Tables 3 and 4. A further component listed as FA-ConAlc in Tables 3 and 4 had a relative retention time of 1·939 (fully silylated trimethyl silyl derivative) and was apparently the same compound found earlier in cell walls of tropical grass stems (Ford and Hartley 1989b). In this earlier work, mass spectrometric examination suggested that the compound may be a substituted cyclobutane dimer formed from ferulic acid and coniferyl alcohol. The total amounts of dimers in the walls were much less than total monomers as can be seen from the ratio of total dimers:total monomers varying from 0·07 to 0·18. The amounts of dimers in the legumes were detectable but lower than in the graminaceous plants.

It seems likely that the substituted cyclobutane dimers are formed during plant growth by photochemical reactions in sunlight involving *p*-coumaroyl and feruloyl groups linked to the cell wall polysaccharides (Hartley and Ford 1989). There is also evidence suggesting that dehydrodiferulic ('diferulic') acid is formed in the cell walls of the growing plant from feruloyl groups by enzyme-catalysed dehydrogenation (Markwalder and Neukom 1976). If it is assumed that dehydrodiferulic acid and the substituted cyclobutane dimers PCA–PCA, PCA–FA and FA–FA (Tables 3 and 4) are formed during plant growth by these mechanisms, then the proportion of original *p*-coumaroyl and feruloyl groups converted to these dimers ranged from 5% (sorghum stem) to 14% (sorghum leaf blade plus sheath). If the dimers cross-

link polysaccharide chains, as has been suggested (Markwalder and Neukom 1976; Hartley and Ford 1989), then the amounts found may be sufficient to limit wall biodegradability.

ACKNOWLEDGEMENTS

The authors are grateful to Dr I Mueller-Harvey for her support, and one of us (F E) would like to thank OECD for a Research Fellowship.

REFERENCES

Akin D E 1989 Plant structure and forage quality. *Agron J* **81** 17–25.

Cherney J H, Axtell J D, Hassen M M, Anliker K S 1988 Forage quality characterization of a chemically induced brown-midrib mutant in pearl millet. *Crop Sci* **28** 783–787.

Cohen M D, Schmidt G M J, Sonntag F I 1964 Topochemistry. Part II. The photochemistry of *trans*-cinnamic acids. *J Chem Soc* 2000–2013.

El-basyouni S Z, Neish A C, Towers G H N 1964 The phenolic acids in wheat. III. Insoluble derivatives of phenolic cinnamic acids as natural intermediates in lignin biosynthesis. *Phytochemistry* **3** 627–639.

Ford C W, Hartley R D 1989a GC–MS characterization of cyclodimers from *p*-coumaric and ferulic acids by photodimerization—a possible factor influencing cell wall biodegradability. *J Sci Food Agric* **46** 301–310.

Ford C W, Hartley R D 1989b Cyclodimers of *p*-coumaric and ferulic acids in the cell walls of tropical grasses. *J Sci Food Agric* **50** 29–43.

Hartley R D 1972 *p*-Coumaric and ferulic acid components of cell walls of ryegrass and their relationships with lignin and digestibility. *J Sci Food Agric* **23** 1347–1354.

Hartley R D 1983 Degradation of cell walls of forages by sequential treatment with sodium hydroxide and a commercial cellulase preparation. *J Sci Food Agric* **34** 29–36.

Hartley R D, Ford C W 1989 Phenolic constituents of plant cell walls and wall biodegradability. In: *Plant Cell Wall Polymers*, eds Lewis N G & Paice M G. Amer Chem Soc Ser 399. American Chemical Society, Washington, DC.

Hartley R D, Jones E C 1975 Effect of ultraviolet light on substituted cinnamic acids and the estimation of their *cis* and *trans* isomers by gas chromatography. *J Chromatogr* **107** 213–218.

Hartley R D, Jones E C 1976 Diferulic acid as a component of cell walls of *Lolium multiflorum*. *Phytochemistry* **15** 1157–1160.

Hartley R D, Jones E C 1977 Phenolic components and degradability of cell walls of grass and legume species. *Phytochemistry* **16** 1531–1534.

Hartley R D, Jones E C 1978a Effect of aqueous ammonia and other alkalis on the *in vitro* digestibility of barley straw. *J Sci Food Agric* **29** 92–98.

Hartley R D, Jones E C 1978b Phenolic components and degradability of cell walls of the brown midrib mutant, bm_3, of *Zea mays*. *J Sci Food Agric* **29** 777–789.

Hartley R D, Jones E C, Fenlon J C 1974 Prediction of the digestibility of forages by treatment of their cell walls with cellulolytic enzymes. *J Sci Food Agric* **25** 947–954.

Hartley R D, Whatley F R, Harris P J 1988 4,4′-Dihydroxytruxillic acid as a component of cell walls of *Lolium multiflorum*. *Phytochemistry* **27** 349–351.

Kahnt G 1967 *Trans–cis* equilibrium of hydroxycinnamic acids during irradiation of aqueous solutions at different pH. *Phytochemistry* **6** 755–758.

Kato A, Azuma J, Kishijima T 1983 A new ferulolylated trisaccharide from bagasse. *Chem Lett (Japan)* 137–140.

Kato Y, Nevins D J 1985 Isolation and identification of O-(5-O-feruloyl-α-L-arabinofuranosyl)-(1→3)-O-β-D-xylopyranosyl-(1→4)-D-xylopyranose as a component of *Zea* shoot cell walls. *Carbohydr Res* **137** 139–150.

Khan R O 1966 *Organic Photochemistry*. McGraw-Hill, New York, p 157.

Kuc J, Nelson O E 1964 The abnormal lignins produced by the brown midrib mutants of maize. I. The brown midrib-I mutant. *Arch Biochem Biophys* **105** 103–113.

Markwalder H U, Neukom H 1976 Diferulic acid as a possible cross-link in hemicelluloses from wheat endosperm. *Phytochemistry* **15** 836–837.

Mueller-Harvey I, Hartley R D, Harris P J, Curzon E H 1986 Linkage of *p*-coumaroyl and feruloyl groups to cell wall polysaccharides of barley straw. *Carbohydr Res* **148** 71–85.

Schmidt G M J 1964 Topochemistry. Part III. The crystal chemistry of some *trans*-cinnamic acids. *J Chem Soc* 2014–2021.

Van Soest P J, Wine R H 1967 Use of detergents in the analysis of fibrous feeds. IV. Determination of plant cell wall constituents. *J Assoc Offic Anal Chem* **50** 50–55.

J Sci Food Agric 1990, **51**, 171–177

Determination of Phosphorus Status of Wheat and Barley Crops Using a Rapid Root Bioassay

A F Harrison and J Dighton

Merlewood Research Station, Institute of Terrestrial Ecology, Grange-over-Sands, Cumbria LA11 6JU, UK

(Received 21 March 1989; revised version received 14 June 1989; accepted 8 August 1989)

ABSTRACT

A root bioassay of soil P availability, based on the amount of ^{32}P-labelled phosphorus taken up in 15 min from a solution in the laboratory, has been tested on seedlings of winter wheat (Triticum aestivum *L*) *and spring barley* (Hordeum vulgare *L*). *The seedlings were taken in spring from six different fertiliser plots, selected to give an extreme range of P availability, from the Broadbalk and Hoosfield experiments at Rothamsted. The results showed a negative relationship between the rate of phosphorus uptake and (a) the phosphorus contents of plants, and (b) Olsen's bicarbonate extractable phosphorus of the soils. This pattern is consistent with the results previously obtained when determining the phosphorus status of trees and grasses. This rapid bioassay procedure may therefore be applicable to agricultural crops.*

Key words: P nutrition, cereal, ^{32}P, roots, bioassay.

INTRODUCTION

Traditionally, the assessment of the phosphorus (P) status of a crop, and therefore the fertiliser requirement, is made from soil analyses. Values of extractable P content of soils relate only to the conditions under which the extraction was carried out and may not exactly mirror what is truly available for plant uptake. Furthermore these analyses have to be evaluated in terms of known crop demands; the analysis alone has only limited value. A method which would integrate both P availability in the soil and the crop demand, particularly if rapid and economic to apply, could therefore be a useful development.

A root bioassay has been developed for the assessment of the P status (degree of

J Sci Food Agric 0022-5142/90/$03.50

deficiency) of forest trees and their fertiliser requirement. The bioassay is based on the rate at which roots take up ^{32}P-labelled P metabolically (Harrison and Helliwell 1979) from a standardised solution in the laboratory; the rate of uptake by roots is negatively related to the P supply and tree productivity, in both pot experiments and in the field (Harrison and Helliwell 1979; Dighton and Harrison 1983). The rate of ^{32}P uptake in the bioassay appears to provide an integrated measure of the balance between the demand for P by the plant and the supply of P in the soil (Harrison and Helliwell 1979). This bioassay has also been found to be applicable to grasses and to the productivity of grasslands on poor upland soils (Harrison *et al* 1986a,b).

As a first test of its potential for use on agricultural crops grown on more fertile soils, the P bioassay has been applied to wheat and barley from certain contrasting plots of the classical long-term experiments on Broadbalk and Hoosfield, Rothamsted. The results of this preliminary study are presented in this paper.

MATERIALS AND METHODS

Seedlings and fertiliser treatments

Winter wheat (*Tritium aestivum* L, Broadbalk) and spring barley (*Hordeum vulgare* L, Hoosfield) plants were sampled in mid-May 1986; seed had been sown on 3 October 1985 and 17 March 1986, respectively. Ten plants (only part of their root systems were recovered) were dug from three random subplots in each of six different fertiliser treatments, which were (a) control with no fertiliser, (b) PK (P at 35 kg ha^{-1}; K at 90 kg ha^{-1}), (c) N2 (N at 96 kg ha^{-1}), (d) N2P (N at 96 kg ha^{-1}; P at 35 kg ha^{-1}), (e) N2PK (N at 96 kg ha^{-1}; P at 35 kg ha^{-1}; K at 90 kg ha^{-1}), and (f) farmyard manure at 35 t ha^{-1}. These treatments were chosen to give a wide range of soil fertility. Full details of the Broadbalk and Hoosfield experiments are given elsewhere (Warren and Johnston 1967; Dyke *et al* 1983). The plants were placed between wet tissue paper in trays, transported back to the laboratory at Merlewood the same day as collected and processed through the bioassay the day following.

P-deficiency bioassay

The roots were processed according to the procedure detailed in Harrison and Helliwell (1979) and Harrison *et al* (1984). Roots were washed carefully, and placed for 30 min in 5×10^{-4} M $CaSO_4$ solution to maintain cell membrane integrity and leach out physically sorbed P in the root free-space. Roots were then transferred to a solution containing the same concentration of $CaSO_4$, 5×10^{-6} M KH_2PO_4 and *c* 1·1 MBq (30 μCi) ^{32}P (as orthophosphate) $litre^{-1}$ at 18 C for 15 min. The root samples were washed under running water for 5 min, and subsamples were counted in 15 ml water in a counting vial by Cerenkov light in a Packard 2000 CA liquid scintillation spectrometer. After this, the root subsamples were removed from the vial, blotted and weighed, and the vial was recounted. The counting efficiency (CE %) was determined using two regressions, one for colour-quench correction and the other for physical quench correction. Further corrections were made for

background counts and isotope decay. Uptake of ^{32}P-labelled P by root samples was calculated in terms of pg P mg wet weight root 15 min^{-1}, with reference to the determined specific activity of the labelled solution.

Chemical analysis

The shoot tissue of each individual plant was dried at 80°C and weighed, and its P and N contents were determined after digestion in conc H_2SO_4/H_2O_2. Soil P availability measurements using the Olsen's bicarbonate (pH 8·5) extraction, for each of the plots sampled, were provided for us (Poulton P R pers comm).

RESULTS AND DISCUSSION

There were marked differences in the rates of ^{32}P-labelled P uptake by the roots of both the wheat and barley, with the rates declining as the soil fertility level and plant productivity increased (Tables 1 and 2). It was not, however, possible to analyse

TABLE 1
Mean rates of ^{32}P-labelled P uptake (±SE) by roots of wheat (W) and barley (B) from the Broadbalk and Hoosfield experiments

Treatment	*Phosphorus uptake*	
	W	*B*
	(*pg P mg*$^{-1}$ *root 15 min*$^{-1}$)	
N2	532±28	1370±177
Control	219±11	751±47
N2P	230±13	694±97
N2PK	195±8	551±20
PK	167±10	430±59
FYM	138±5	389±24

TABLE 2
Mean dry weights and P and N contents (±SE) of wheat and barley shoots[a] from Broadbalk and Hoosfield

Treatment	*Wheat*			*Barley*		
	Wt (mg)	*P (μg)*	*N (μg)*	*Wt (mg)*	*P (μg)*	*N (μg)*
N2	142±17	65±7	753±82	53±3	12±1	229±13
Control	273±24	122±12	629±45	90±6	27±2	268±33
N2P	508±50	280±24	2089±187	108±8	57±4	585±46
N2PK	727±103	357±47	2505±330	150±9	96±6	756±51
PK	372±33	182±14	830±85	144±11	97±13	449±41
FYM	1983±214	832±77	3720±330	327±27	192±30	1058±102

[a] Roots were only partially recovered from the soil, so shoot data only are compared.

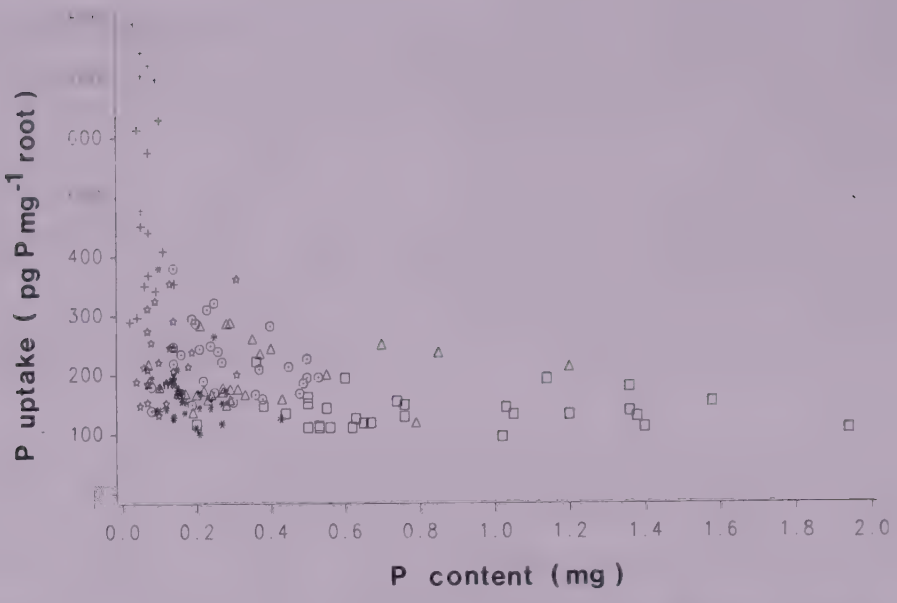


Fig 1. Relationship between the uptake of ^{32}P-labelled P by roots and the P content (mg per plant) of shoots of winter wheat. Individual plant measurements plotted for all six fertiliser treatments. ☆ = Control, unfertilised; * = PK; + = N2; ⊙ = N2P; △ = N2PK; □ = FYM.

these data statistically, eg by analysis of variance, as the classical experiments do not have replicate plots. The variability between the sub-plot root samples was low, supporting the conclusion that there were significant treatment differences. The mean rates of ^{32}P-labelled P uptake in the bioassay by roots of both crops were negatively related to the total P content of plants when expressed as mg P per plant (Tables 1 and 2), as found for tree seedlings (Harrison and Helliwell 1979). These results extend the findings of earlier research that low P status barley plants show higher rates of P uptake by roots than those of plants grown with higher P status (Humphries 1951; Cartwright 1972; Clarkson *et al* 1978).

The wheat plants had, in general, lower average rates of ^{32}P-labelled P uptake by roots than the barley, and this is reflected in the average amounts and concentrations of total P present in the seedlings; the winter wheat had grown for longer than the barley and had therefore been able to acquire a greater quantity of P from the soil.

When the ^{32}P-labelled P uptake value was plotted against the mg total P content for all individual plants, irrespective of the fertiliser treatment (Figs 1 and 2), there was a negative curvilinear (asymptotic) relationship between these two parameters for both the wheat and the barley, though the points were less spread along the *x*-axis with barley because of its lower plant total P content. A similar pattern was found between ^{32}P-labelled P uptake values and plant nitrogen content, expressed as mg total N per plant. From Figs 1 and 2 it can be seen that at very low plant total P contents (eg N2 treatment) there is a wide range of high ^{32}P-labelled P uptake values, whereas at high plant total P contents (FYM treatment) there was little change in the ^{32}P-labelled P uptake values. These findings indicate a greater sensitivity of the bioassay technique under low P supply conditions.

This relationship is very similar to the relationships between ^{32}P-labelled P uptake in the bioassay and P supply in the soil found for tree seedlings and grasses (Harrison and Helliwell 1979; Harrison *et al* 1986a). The ^{32}P-labelled P uptake

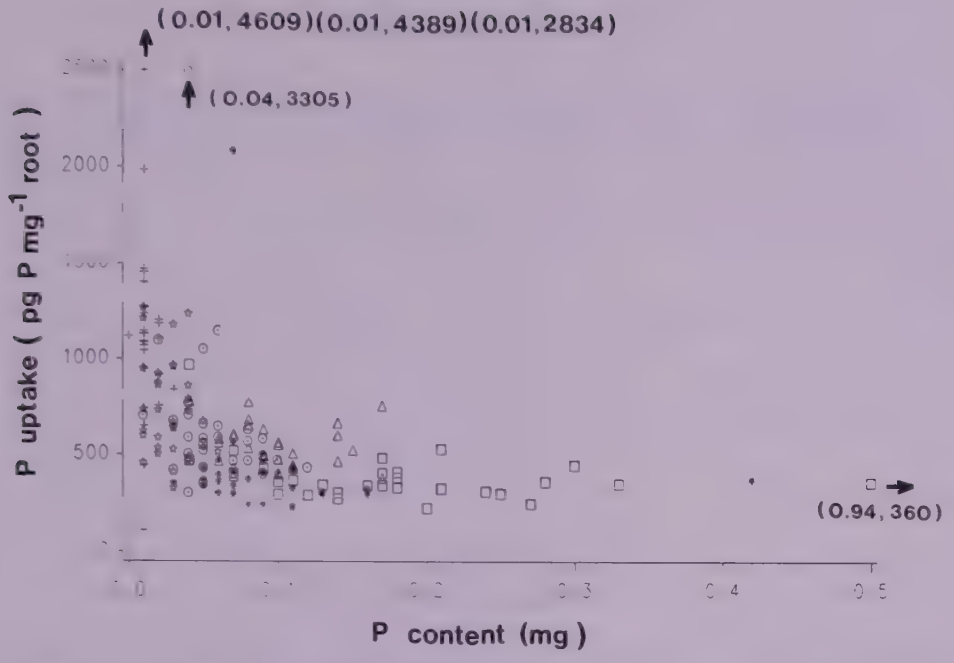


Fig 2. Relationship between the uptake of ^{32}P-labelled P by roots and the P content (mg per plant) of shoots of barley. Individual plant measurements plotted for all six fertiliser treatments. Treatments distinguishable by symbols as in Fig 1.

TABLE 3

Availability (Olsen; 0·5 M $NaHCO_3$) of phosphorus in the top soils[a] of selected plots from the Broadbalk and Hoosfield experiments. Data were provided by P R Poulton (Rothamsted Experimental Station)

Treatment	*Extractable P in μg g^{-1} soil*	
	Broadbalk 1987	*Hoosfield 1982*
N2	2	4[b]
Control	4	9
N2P	70	102
N2PK	98	133
PK	72	156
FYM	87	124

[a] The Broadbalk plots contain 2910 t soil ha^{-1} (weight of oven-dry soil < 6·25 mm to a depth of 23 cm), except for the FYM plot, which contains 2300 t ha^{-1}. The Hoosfield plots contain 2620 t ha^{-1}, except for the FYM plot which contains 2290 t ha^{-1}.

[b] Value for the N2K treatment.

values also relate negatively to estimates of P supply in the Broadbalk and Hoosfield plots, as shown by the quantities of P extractable in Olsen bicarbonate (0·5 M at pH 8·5) solution (Table 3; Fig 3); adjustment of the extractable P values to allow for the small differences in soil bulk densities makes little difference to the overall picture. Furthermore, though the soils had been sampled in September 1987

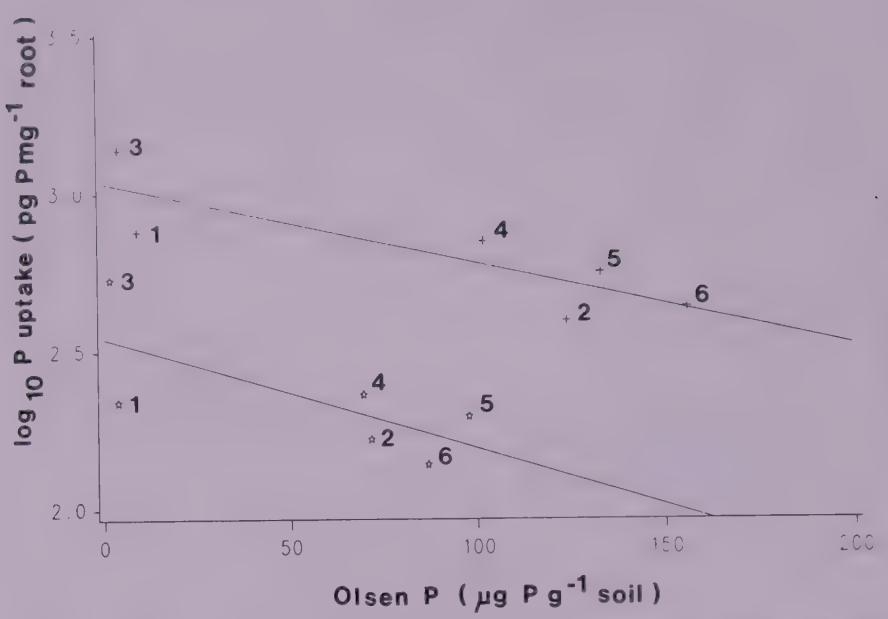


Fig 3. Relationships between the $\log_{10}$ mean uptake rate of ^{32}P-labelled P by roots of wheat (☆) and barley (+) and the Olsen's bicarbonate extractable soil P from the six fertiliser treatments (1 = control, unfertilised; 2 = PK; 3 = N2; 4 = N2P; 5 = N2PK; 6 = FYM). Regression equations are:

Wheat – $Y = 3{\cdot}03 - 0{\cdot}0026x$ $\quad r = -0{\cdot}85$
Barley – $Y = 2{\cdot}54 - 0{\cdot}0035x$ $\quad r = -0{\cdot}72$

and thus may have shown some seasonal variation, the differences in P availability between the treatments in the long-term experiments are likely to have been very similar.

Perhaps the most interesting comparisons are those between the control (unfertilised) treatment and the N-only treatment, for both wheat and barley. The rates of P uptake in the bioassay by roots of the N-only treatments are considerably higher than those of the control plants (Table 1), reflecting the increased P deficiency brought about by repeated application of the nitrogen; the N-only plants are also smaller and contained less P than the control plants (Table 2). Similarly there is a contrast between the N2 treatment and the N2P and N2PK treatments, both of which gave good growth and balanced P and N contents of the plants.

The findings reported here parallel those that we have found for trees and grasses. These preliminary results indicate that this bioassay warrants further investigation for application to agricultural crops. The procedure is easy, sensitive and rapid to carry out, though it requires the use of radiophosphorus and appropriate laboratory facilities. The same also applies to the potassium bioassay (Jones *et al* 1987) and the nitrogen bioassay currently under research development.

ACKNOWLEDGEMENTS

We wish to thank D S Jenkinson for his interest, P R Poulton for the Olsen P measurements, and the authorities ar Rothamsted Experimental Station (IACR) for permission to sample the long-term experiments.

REFERENCES

Cartwright B 1972 The effect of phosphate deficiency on the kinetics of phosphate absorption by sterile excised barley roots, and some factors affecting the ion uptake efficiency of roots. *Comm Soil Sci Pl Anal* **3** 313–322.

Clarkson D T, Sanderson J, Scattergood C 1978 Influence of phosphate-stress on phosphate absorption and translocation by various parts of the root system of *Hordeum vulgare* L. (barley). *Planta* **139** 47–53.

Dighton J, Harrison A F 1983 Phosphorus nutrition of lodgepole pine and Sitka spruce stands as indicated by a root bioassay. *Forestry* **56** 33–43.

Dyke G V, George B J, Johnston A E, Poulton P R, Todd A D 1983 The Broadbalk Wheat Experiment 1968–78: yields and plant nutrients in crops grown continuously and in rotation. *Rothamsted Exp Sta Rept 1982 Pt 2*, pp 5–44.

Harrison A F, Helliwell D R 1979 A bioassay for comparing the availability of phosphorus in soils. *J Appl Ecol* **16** 497–505.

Harrison A F, Dighton J, Smith M R 1984 The phosphorus deficiency bioassay: sample and data handling procedures. *Merlewood Research and Development Paper No 103*.

Harrison A F, Dighton J, Hatton J C, Smith M R 1986a A phosphorus-deficiency bioassay for trees and grasses growing in low nutrient status soils. In: *Proc VIth Int Coll Optim Plant Nutr*, September 1984, Vol 3, ed Martin-Prevel P. IAONP, Montpellier, pp 957–963.

Harrison A F, Hatton J C, Taylor K 1986b Application of a root bioassay for determination of P-deficiency in high-altitude grasslands. *J Sci Food Agric* **37** 10–11.

Humphries E C 1951 The absorption of ions by excised root systems. II Observations on roots of barley grown in solutions deficient in phosphorus, nitrogen or potassium. *J Exp Bot* **2** 344–379.

Jones H E, Harrison A F, Dighton J 1987 A ^{86}Rb bioassay to determine the potassium status of trees. *New Phytol* **107** 695–708.

Warren R G, Johnston A E 1967 Hoosfield continuous barley. *Rothamsted Exp Sta Rept 1966 Pt 2*, pp 320–338.

J Sci Food Agric 1990, **51**, 179–183

Effect of 2,2′-Dipyridyl on Silage Fermentation

Bart J Bruyneel, Marleen M Vande Woestyne and Willy H Verstraete*

Laboratory of Microbial Ecology, Faculty of Agricultural Sciences, State University of Gent, Coupure L 653, B-9000 Gent, Belgium

(Received 2 May 1989; revised version received 5 July 1989; accepted 30 August 1989)

ABSTRACT

The fermentation of grass can be altered by the addition of 2,2′-dipyridyl so that a lactic acid fermentation occurs rather than a putrefying fermentation (butyric acid fermentation). The combined addition of 2,2′-dipyridyl and inoculation with Lactobacillus plantarum *is even more favourable and results in high quality silage. 2,2′-Dipyridyl is too toxic for general use, but the results of laboratory-scale studies suggest that other sequestrants of iron(II) ions warrant further research with respect to their capability to enhance lactic acid fermentations.*

Key words: Silage, 2,2′-dipyridyl, *Lactobacillus plantarum*, iron complexation.

INTRODUCTION

The aim of ensilage is to conserve forage in times of surplus for times of shortage (McDonald 1981) through a natural lactic acid fermentation. The success of this fermentation depends on a number of parameters such as buffering capacity, availability of sugars, water content, soil contamination and bacterial densities of different organisms. Clostridia and enterobacteria are spoiling microorganisms, due to their action on proteins and lactic acid. Their growth gives rise to off flavours from butyric acid, cadaverine and putresceine, and also to dry matter losses (Beck 1978; McDonald 1981; Woolford 1984). Yeasts ferment sugars and lactate to ethanol, which can cause severe diseases in animals (Vetter and Von Glan 1978). To

* To whom correspondence should be addressed.

J Sci Food Agric 0022-5142/90/$03.50

avoid malfermentation in silage, additives are often used. Such additives can be fermentation inhibitors (such as acids and bacteriocides) as well as fermentation enhancers (such as lactic acid bacteria, sugars and enzymes) (Lusk 1978; Arnould 1981; Woolford 1984).

In this laboratory-scale study, a synthetic iron(II) scavenger was used as a model compound to direct the microbial fermentation during ensilage of grass. Only lactic acid bacteria are able to grow in the absence of iron (Macham 1976). The addition of 2,2′-dipyridyl and the concomitant scavenging of all available iron should therefore inhibit the development of yeasts, clostridia and enterobacteria. Accordingly lactic acid bacteria should be enhanced in their development, which should result in a faster pH decline and a stable silage.

MATERIALS AND METHODS

In a first experiment, Italian ryegrass (DM 20%) which had been fertilised with 120 kg N ha^{-1} was mixed with 10 g $CaCO_3$ and 10 g sandy loam soil per kg grass (wet weight) and ensiled in dark brown bottles (250 ml). A waterlock was installed to allow the gas to escape without interfering with the anaerobic conditions. The addition of $CaCO_3$ and sandy loam soil made it possible to create adverse silage conditions respectively by increasing the buffering capacity and by the addition of undesirable microorganisms. Besides the control, a treatment was included with 1 g kg^{-1} of 2,2′-dipyridyl. Each treatment was repeated five times to allow statistical evaluation. After 3 months at room temperature, all bottles were analysed for lactic acid, volatile fatty acids and pH.

The experiment was repeated with Italian ryegrass fertilised with 800 kg N ha^{-1} and a DM content of 25%. Grass fertilised with such a high amount of N invariably produces low quality silages (Spoelstra 1983). The grass was also mixed with 10 g $CaCO_3$ and 10 g sandy loam soil per kg grass before ensiling. In addition to the control, a 1 g kg^{-1} 2,2′-dipyridyl treated silo, a treatment with 10^5 lactobacilli g^{-1} of grass and a treatment with 1 g kg^{-1} 2,2′-dipyridyl together with 10^5 lactobacilli g^{-1} grass were prepared. A mixture of six *Lactobacillus plantarum* strains, all isolated from grass, was used for inoculation. The silos were kept at room temperature and analysed after 3 months. For statistical purposes the experiment was done in five replicates. Lactic acid, volatile fatty acids and pH were determined.

Silage (10 g) was mixed thoroughly with 90 ml of demineralised water for pH determination. Lactic acid and volatile fatty acids were extracted from the silage material as follows (Henderson A R pers comm). Silage (10 g) was blended with 90 ml of 0·05 M H_2SO_4 and allowed to stand for 24 h at 4°C. The solution was then filtered through a Whatman No 4 filter. The filtrate was then treated according to the method described by Holdeman and Moore (1972). Lactic acid was analysed using gas chromatography (Hewlett Packard 572, column, 32 mm, Chromosorb GAW DCMS support, 80–100 mesh, flame ionisation detector, N_2 carrier gas, 3380 HP integrator). The volatile fatty acids were also analysed by gas chromatography (Carlo Erba Fractovap 4160, column: FFAP 25 m, Carbowax 20M, N_2 carrier gas, flame ionisation detector).

TABLE 1
Effect of 2,2′-dipyridyl on pH and fatty acid content (g kg^{-1} DM) of grass silage

Treatment		*pH*	*Lactic acid*	*Acetic acid*	*Butyric acid*
Control	*Mean*	5·51[a]	90·1[a]	24·1	20·4[a]
	SD	0·22	41·8	4·6	17·0
Treatment	*Mean*	4·77[b]	154·9[b]	22·5	0·0[b]
	SD	0·13	12·9	3·2	0·0

Different characters (*a*,*b*) indicate a significant difference ($P < 0·05$).
SD is standard deviation.

RESULTS AND DISCUSSION

In the first experiment, the grass had a pH value of 6·95 at the moment of ensiling. After ensiling, the pH of the 2,2′-dipyridyl-treated silage was significantly lower than that of the control (Table 1). Accordingly, the lactic acid content was significantly higher whereas no significant difference could be seen in the acetic acid content (Table 1). No butyric acid was present in the 2,2′-dipyridyl treated silage, and up to 20·4 g kg^{-1} DM was found in the control (Table 1).

Table 1 clearly shows that the butyric acid fermentation ceased completely as a result of the addition of the iron(II) scavenger, even when the conditions were adverse for the lactic acid bacteria. The Flieg value, ie a value based on the fatty acid composition and indicative of silage quality (Woolford 1984), was 67 points for the control whereas the optimal value of 100 was reached in the 2,2′-dipyridyl-treated silage. A consequence of the use of iron(II) scavengers in silage is that, due to the complexation reaction, the grass becomes a reddish colour.

In the second experiment the initial pH of the grass was 7·1. The pH change was significantly different depending on the treatment used. The lowest pH was obtained when 2,2′-dipyridyl was used together with a mixed inoculation of *Lactobacillus plantarum* (Table 2). When 2,2′-dipyridyl alone was added, the pH did not drop below 5·0. Inoculation seemed to increase the efficiency of the silage fermentation. The production of both lactic acid and acetic acid was significantly higher when 2,2′-dipyridyl was present and when *L plantarum* was added. The acetic acid content and the pH in the 2,2′-dipyridyl-treated silages without the addition of *L plantarum* were considerably higher than when inoculation occurred. Addition of *L plantarum* strains resulted in a higher lactic acid content in comparison with the absence of inoculum. This again indicated the increased efficiency of silaging through inoculation. High levels of butyric acid were present in the untreated silages. A mean of 45·3 g butyric acid was present per kg of silage DM when no treatment was provided (Table 2). Only 2·5 g butyric acid kg^{-1} DM was present when 2,2′-dipyridyl was added. Inoculation with *L plantarum* also appeared to be efficient since only 0·5 g butyric acid kg^{-1} DM could be detected. Butyric acid was absent when *L plantarum* together with 2,2′-dipyridyl was added to the silage.

TABLE 2
Effect of different treatments on pH and fatty acid composition (g kg^{-1} DM) of grass silage

Treatment		*pH*	*Lactic acid*	*Acetic acid*	*Butyric acid*
Control	*Mean*	$5{\cdot}99^{a}$	$21{\cdot}5^{a}$	$3{\cdot}6^{a}$	$45{\cdot}3^{a}$
	SD	0·22	8·1	0·3	8·2
L plantarum	*Mean*	$4{\cdot}60^{b}$	$105{\cdot}2^{b}$	$21{\cdot}6^{b}$	$0{\cdot}5^{b}$
	SD	0·03	15·9	2·1	0·7
2,2′-Dipyridyl	*Mean*	$5{\cdot}19^{c}$	$81{\cdot}1^{c}$	$24{\cdot}7^{b}$	$2{\cdot}5^{b}$
	SD	0·11	13·6	4·0	3·0
L plantarum + 2,2′-dipyridyl	*Mean*	$4{\cdot}39^{d}$	$122{\cdot}1^{b}$	$18{\cdot}0^{c}$	$0{\cdot}0^{b}$
	SD	0·04	20·9	2·3	0·0

Different characters (*a*,*b*,*c*,*d*) indicate a significant difference ($P < 0{\cdot}05$).
SD is standard deviation.

CONCLUSION

A silage fermentation can be oriented towards a dominantly lactic acid fermentation by the addition of 2,2′-dipyridyl. Optimal results were obtained by a combination of 2,2′-dipyridyl treatment with an *L plantarum* inoculation.

In this study, 2,2′-dipyridyl was used as a model for iron complexation. However, 2,2′-dipyridyl is not acceptable as a feed additive owing to its toxicity (LD_{50} mice intraperitoneal = 200 mg kg^{-1}). More generally, it can be stated that iron(II) scavengers open interesting perspectives for microbial eco-physiology in general. Research to develop suitable iron(II) sequestrants compatible with technological and toxicological requirements appears justified.

ACKNOWLEDGEMENT

The technical skill of S Vonckx and the financial support of the RADAR company are gratefully acknowledged.

REFERENCES

Arnould R 1981 Bewarrmiddelen voor kuilvoeder. *Landbouwtijdschrift* **34** 749–762.

Beck Th 1978 The microbiology of the silage fermentation. In: *Fermentation of Silage—a Review*, ed McCullough M E. National Feed Ingredients Association, West Des Moines, Iowa, pp 61–116.

Holdeman L V, Moore W E C 1972 *Anaerobe Laboratory Manual*. The Virginia Polytechnic Institute and State University Anaerobe Laboratory, Blacksburg, VA.

Lusk J W 1978 The use of preservatives in silage production. In: *Fermentation of Silage—a Review*, ed McCullough M E. National Feed Ingredients Association, West Des Moines, Iowa, pp 201–232.

Macham L 1976 Iron and micro-organisms. *Process Biochem* **11** 12–17.
McDonald P 1981 *The Biochemistry of Silage*. Wiley, New York.
Spoelstra S F 1983 Inhibition of clostridial growth by nitrate during the early phase of silage fermentation. *J Sci Food Agric* **34** 145–152.
Vetter R L, Von Glan K N 1978 Abnormal silages and silage related disease problems. In: *Fermentation of Silage—a Review*, ed McCullough M E. National Feed Ingredients Association, West Des Moines, Iowa, pp 281–332.
Woolford M K 1984 *The Silage Fermentation*. Marcel Dekker, New York.

J Sci Food Agric 1990, **51**, 185–192

Analysis of Carbohydrates in Seven Edible Fruits of Bangladesh

Nilufar Nahar, Shakila Rahman and M Mosihuzzaman

Department of Chemistry, University of Dhaka, Dhaka-1000, Bangladesh

(Received 22 February 1989; revised version received 11 May 1989; accepted 8 August 1989)

ABSTRACT

Dry matter, ash, lignin, starch and soluble and insoluble dietary fibre contents of the edible parts of seven fruits of Bangladesh were determined. Analysis of the low molecular weight carbohydrates showed that all the fruits, except lukluki and hogplum, contained substantial amounts of these materials of which glucose and fructose were the main components. The main constituent of the polysaccharides in all the fruits was glucose. The dietary fibre contents of the dry fruits ranged from 29% to 79%. Lukluki has by far the best combination of low free sugars and high dietary fibre, and pineapple the worst.

Key words: Carbohydrates, free sugars, dietary fibre (DF), soluble DF, insoluble DF, mango (*Mangifera indica* L), pineapple (*Ananas comosus* L), guava (*Psidium guajava* L), hogplum (*Spondias dulcis* L), kamranga (*Averrhoa carambola* L), latkan (*Baccaurea ramiflora* (Lour)), lukluki (*Flacourtia indica* Burm f).

INTRODUCTION

The carbohydrates in fruits are important in that fruits containing high proportions of free sugars are restricted for diabetic patients. However, polymeric carbohydrates forming the major part of the dietary fibre (DF) are beneficial to both diabetic and heart patients because DF lowers (Trowell 1986) both blood sugar and serum cholesterol. Although the functions of DF are yet to be specified, its importance in human nutrition has been well recognised (Furda 1987) and fruits are a very convenient source of DF.

A very wide variety of fruits grow in tropical Bangladesh where food is in short

J Sci Food Agric 0022-5142/90/$03.50

supply and the nutrition level is low. Very little is known about the sugar and DF composition of Bangladesh fruits especially the endemic ones. A preliminary report (Ahmed *et al* 1986) lists vitamin, protein and total carbohydrate contents of some of the local fruits. Studies of sugar (Wali and Hasan 1965; Chan and Kwok 1975a) and DF (Wills *et al* 1986; Candlish *et al* 1987) of guava, pineapple, kamranga and mango, which grow in many tropical countries, have recently been reported. Analyses of a polysaccharide from mango seed (Amin and Sayeed 1973) and an arabinan from guava (Sengupta *et al* 1965) were reported earlier. However, the polysaccharides in DF of these fruits have not been reported. As a part of our studies of carbohydrates in local fruits and vegetables for evaluating their nutritional status, we are analysing the local cultivars of the more common tropical fruits as well as those which are exclusively native to Bangladesh. We now report the analyses of low molecular weight carbohydrates (including free sugars) and DF of seven important fruits of Bangladesh.

MATERIALS AND METHODS

Fruit material

Good quality ripe fruits (Table 1) were bought from the Dhaka market during 1986. Edible parts (pulp in the case of mango and pineapple; pulp and skin in all other cases) were carefully separated from the rest of the fruit material. The fruits (edible parts) were dried at 40°C and ground in a Cyclotec grinding machine with a 0·5-mm screen. Several fruits of each type (a minimum of three) were taken for analyses.

General methods

Evaporations were conducted under reduced pressure at ≤ 40°C. GLC was conducted with a Pye-Unicam 4500 or Packard 427 instrument fitted with flame ionisation detector and quartz capillary columns (25 m or 12·5 m × 0·2 mm id). Separations were performed on Cp sil 5 (TMS derivatives) or Cp sil 88 (acetates and alditol acetates) WCOT columns. The peak areas were calculated with a Hewlett-Packard 3390A integrator. All results are averages of at least two determinations.

TABLE 1
Composition of the fruits (% of dry matter)

Botanical name	*Common name*	*Dry matter (%)*	*Ash*	*Lignin*	*Protein*
Mangifera indica (L)	Mango	18·9	4·2	14·2	4·1
Ananas comosus (L)	Pineapple	9·7	0·1	3·0	2·0
Psidium guajava (L)	Guava	13·3	3·6	22·5	3·5
Spondias dulcis (L)	Hogplum	12·5	7·7	5·9	5·0
Averrhoa carambola (L)	Kamranga	5·9	3·0	11·2	7·5
Baccaurea ramiflora (Lour)	Latkan	14·7	5·8	8·2	2·1
Flacourtia indica (Burm f)	Lukluki	51·2	2·3	23·7	7·2

Determination of dry matter, ash, protein, lignin, total polysaccharides and uronic acid

Dry matter and ash contents were determined following standard procedures (Theander and Westerlund 1986). Protein contents were calculated from elemental nitrogen analysis (N × 6·25). For the determination of polysaccharide constituents and lignin, the fruit powder was hydrolysed with sulphuric acid (12·0 M at room temperature for 2 h followed by refluxing for 6 h after dilution to 0·36 M, Saeman *et al* 1954). The neutral sugar constituents were analysed (Sawardeker *et al* 1965) by GLC as their alditol acetates. The insoluble residue was determined gravimetrically as Klason lignin. The uronic acid contents were determined by a decarboxylation method (Bylund and Donetzhuber 1968).

Analysis of free sugars

Fresh edible parts (500 g) of the fruits were refluxed for 30 min with enough ethanol to keep the water concentration to about 20%. The extraction with aqueous 80% ethanol was repeated two more times. The combined extract was evaporated to a small volume (400 ml), diluted to 600 ml and extracted (3 × 400 ml) with chloroform. The water solution was concentrated to a small volume and passed through Dowex 50 W × 8 H^+ and Amberlite CG 400 OH^- ion exchange columns (Theander and Åman 1976). The neutral fractions (5 mg) with allitol (0·2 mg) as internal standard were converted to trimethylsilyl (TMS) ethers and analysed by GLC (Sweeley *et al* 1963).

Determination of soluble and insoluble DF contents

Each dried and powdered fruit sample (1 g) was extracted by refluxing (3 × 75 ml, 30 min each time) with aqueous 80% ethanol followed by chloroform. The residue was dried at 40° C, suspended in acetate buffer (75 ml, 0·1 M, pH 5·0) and treated with α-amylase (Termamyl type 120L, Novo A/S, Copenhagen, Denmark) at 96° C for 1 h. After cooling, the mixture was incubated for 16 h at 60° C with amyloglucosidase (2 ml suspension, from *Aspergillus niger*, Boehringer Mannheim, Mannheim, FRG). The cooled suspension was centrifuged and the insoluble residue was suspended in water, dialysed and freeze dried giving the insoluble DF. The supernate was dialysed and freeze dried giving the soluble DF (Theander and Westerlund 1986).

Determination of starch

Small parts (100 μl) of the supernate from the DF determinations were treated with glucose-oxidase reagent (Boehringer Mannheim), and from the absorbance at 510 nm of the resulting coloured solutions the glucose contents were determined by comparing with a standard glucose curve. The starch contents of the fruits were then calculated (Salomonsson *et al* 1984).

Analysis of neutral sugar constituents of soluble DF

Soluble DF (10 mg) and allose (1 mg, internal standard) were treated with trifluoroacetic acid (5 ml, 2 M) for 3 h at 120° C. The resulting neutral sugars were analysed by GLC as their alditol acetates (Sawardeker *et al* 1965).

RESULTS AND DISCUSSION

Seven local fruits covering a wide range of taste were taken for the present study. The dry matter and lignin contents of the fruits (Table 1) varied to a great extent. The ash contents were rather high except in pineapple. The fruits under investigation contained relatively low amounts of protein.

Free sugars

The major free sugar in mango and pineapple was sucrose (Table 2). Similar findings have been reported earlier (Chan and Kwok 1975a; Wills *et al* 1986). In these reports the total free sugar content of about 12 and 8% for mango and pineapple, respectively, was slightly higher than our values of 8 and 5·8%. Such differences may be due to variations in the species of fruits used. Thus, our free sugar value for guava was 3·1%, that quoted by Wills *et al* (1986) was 3·4%, whereas Chan and Kwok (1975a) found 5·8% of free sugars in guava. The sucrose content in guava and in the rest of the fruits was very low. Wills *et al* (1986) and Chan and Kwok (1975a) have also reported low sucrose content in guava. However, invertase activity, as reported in papaya (Chan and Kwok 1975b) and lychee (Chan *et al* 1975), may also be responsible for the absence or negligible presence of sucrose in these fruits. The reasonably sweet latkan and kamranga had rather low total free sugar (Table 2) compared with mango and pineapple. As expected, the non-sweet hogplum and lukluki had a very low free sugar content. In contrast to other plant materials (Mosihuzzaman *et al* 1982; Nahar *et al* 1989) sugar alcohols were either absent or were present to a small extent in the fruits, except for a notable presence of free glycerol in guava, and glucitol in lukluki. As found in all plant materials, all the fruits contained *myo*-inositol in small proportions.

Total polysaccharides

Analysis of total polysaccharides of the fruits showed these materials were mainly polymers of glucose (Table 3). However, significant amounts of galactose followed by xylose and arabinose were present in all the fruits indicating substantial amounts of non-glucan polymers in these fruits.

Soluble and insoluble DF and starch contents

The DF content, on a dry weight basis, of all the fruits was high (Table 4). Lukluki was significantly different in that it contained a very high proportion (34·5%) of DF on a fresh weight basis. Soluble DF constituted about 10–25% of the total DF content of the fruits except that kamranga contained only about 4% of soluble DF. The starch content of the fruits was very low except for hogplum (18·6%; Table 4).

Analysis of soluble DF

As the insoluble DF was composed mainly of cellulose and lignin, these were not further analysed. Although the role of insoluble and soluble DF may not be distinguished, polysaccharides in soluble DF may be more likely to take part in different biological and metabolic activities.

Analysis of sugar constituents of soluble DF (Table 5) revealed that these were

TABLE 2
Low molecular weight carbohydrates in the fruits

Fruits	*Glycerol*	*Glucose*	*Fructose*	*Mannitol*	*Glucitol*	Myo-*inositol*	*Sucrose*	*Total free sugars (% of fresh fruit)*
			mg g^{-1} fresh fruit					
Mango	0·1	10·4	8·0	0·6	—	1·0	59·4	7·8
Pineapple	0·2	14·0	4·0	—	—	0·8	40·0	5·8
Guava	2·0	20·2	10·1	—	—	0·6	0·8	3·1
Hogplum	0·1	2·0	0·5	—	—	0·8	—	0·3
Kamranga	Trace	7·1	8·6	0·2	0·4	0·2	Trace	1·6
Latkan	0·1	14·0	7·0	0·2	—	0·1	0·7	2·2
Lukluki	0·2	1·0	5·4	—	2·2	0·2	Trace	0·6

TABLE 3
Relative composition of total polysaccharide constituents of the fruits (%)

Fruits	*Rha*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*Uronic acid*
Mango	0·6	5·4	10·1	2·0	18·0	61·7	2·2
Pineapple	0·4	9·5	24·4	1·7	9·1	52·5	2·4
Guava	0·5	6·2	25·5	1·2	9·7	51·5	5·4
Hogplum	0·4	8·5	2·3	0·4	13·1	67·8	7·5
Kamranga	0·5	13·3	7·6	5·1	16·5	51·2	5·8
Latkan	1·2	3·8	13·6	4·8	17·3	58·7	0·6
Lukluki	0·4	6·4	9·9	Trace	27·7	48·1	7·5

TABLE 4
Dietary fibre and starch contents of the fruits

Fruit	*Dietary fibre content*				*Starch (% of dry fruit)*
	Soluble (% of dry fruit)	*Insoluble (% of dry fruit)*	*Total*		
			% of dry fruit	*% of fresh fruit*	
Mango	3·9	33·1	37·0	8·0	0·2
Pineapple	5·5	23·4	28·9	2·8	2·2
Guava	7·0	67·4	74·4	9·9	0·5
Hogplum	10·3	36·9	47·2	5·9	18·6
Kamranga	2·4	69·9	72·3	4·3	0·5
Latkan	20·2	58·8	79·0	11·6	0·5
Lukluki	6·3	64·9	71·2	36·5	0·4

TABLE 5
Relative composition of polysaccharide constituents of soluble dietary fibre (DF) of the fruits (%)

Fruits	*Rha*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*Uronic acid*
Mango	7·9	20·7	21·5	4·6	19·4	15·8	10·1
Pineapple	Trace	4·4	13·5	21·0	32·7	24·4	4·0
Guava	5·9	43·4	3·9	4·0	15·5	16·3	11·0
Hogplum	4·4	21·2	21·3	10·4	21·9	18·3	2·5
Kamranga	2·8	7·5	27·3	14·1	18·1	26·2	4·0
Latkan	0·4	11·8	13·6	4·5	17·2	46·5	6·0
Lukluki	6·7	23·0	7·5	25·2	18·2	8·3	11·1

complex mixtures of polysaccharides. The soluble DF of guava, lukluki, hogplum and mango was rich in arabinose whereas those of pineapple and lukluki were rich in mannose. Glucose, galactose and xylose were present in significant amounts in the soluble DF of all the fruits except that lukluki contained a very small amount of glucose, and guava and lukluki very small amounts of xylose. Fractionation,

purification and structural studies of the polysaccharides and glycoconjugates of the fruits are in progress.

In the case of some of the fruits, our values for free sugars are relatively low, and those for DF contents are high, compared with those reported earlier (Chan and Kwok 1975a; Wills *et al* 1986; Candlish *et al* 1987). Considering the large number of variables involved, eg season, maturity, storage time, extraction procedure and cultivar, these differences are not surprising. From the results of this study attempts may be made to choose more suitable fruits for diabetic and heart patients with a balance between free sugars and DF contents. However, to make the choice more general the same fruit grown at different seasons and climates should be taken into consideration.

ACKNOWLEDGEMENTS

The International Foundation for Science, Stockholm, and the International Program in the Chemical Sciences provided financial support. Professor Olof Theander of the Swedish University of Agricultural Sciences provided some instrumental facilities.

REFERENCES

Ahmed K, Malek M, Jahan K, Salamatullah K 1986 *Nutritive Value of Food Stuff* (3rd edn). Institute of Nutrition and Food Science, University of Dhaka, p 18.

Amin S, Sayeed M M 1973 Polysaccharides of mango seeds. *Carbohydr Res* **27** 39–46.

Bylund M, Donetzhuber A 1968 Semimicro determination of uronic acids. *Svensk Papperstidn* **15** 505–508.

Candlish J K, Gourley L, Lee H P 1987 Dietary fibre and starch in some Southeast Asian fruits. *J Food Comp Anal* **1** 81–84.

Chan H T, Kwok H C M 1975a Identification and determination of sugars in some tropical fruit products. *J Food Sci* **40** 419–420.

Chan H T, Kwok H C M 1975b Importance of enzyme inactivation prior to extraction of sugars from papaya. *J Food Sci* **40** 770–771.

Chan H T, Kwok H C M, Lee C W Q 1975 Sugar composition and invertase activity in lychee. *J Food Sci* **40** 772–774.

Furda I 1987 Beneficial intestinal effects of non-starch polysaccharides. *Industrial Polysaccharides*, eds Stivala S S, Crescenzi V & Dee I C M. Gordon and Breach, New York, pp 195–202.

Mosihuzzaman M, Theander O, Åman P 1982 Analysis of carbohydrates in the jute plant (*Corchorus capsularis*). *J Sci Food Agric* **33** 1207–1212.

Nahar N, Mosihuzzaman M, Theander O 1989 Analysis of phenolic acids and carbohydrates in pigeon pea (*Cajanus cajan*) .plant. *J Sci Food Agric* **50** 45–53.

Saeman J F, Moore W E, Mitchel R L, Millett M A 1954 Techniques for the determination of pulp constituents by quantitative paper chromatography. *Tappi* **37** 336–343.

Salomonsson A C, Theander O, Westerlund E 1984 Chemical characterization of Swedish whole meal and bran fractions. *Swedish J Agric Res* **14** 111–117.

Sawardeker J S, Sloneker J H, Jeanes A R 1965 Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography. *Anal Chem* **37** 1602–1604.

Sengupta U K, Mukherjee A K, Rao C V N 1965 Structural investigation of the arabinan from guava. *Aust J Chem* **18** 851–857.

Sweeley C C, Bentley R, Makita M, Wells W W 1963 Gas–liquid chromatography of trimethylsilyl derivatives of sugar and related substances. *J Amer Chem Soc* **85** 2497–2507.

Theander O, Åman P 1976 Low-molecular carbohydrates in rapeseed and turnip rapeseed meals. *Swedish J Agric Res* **6** 81–85.

Theander O, Westerlund E 1986 Studies on dietary fibre. 3. Improved procedures for analysis of dietary fibre. *J Agric Food Chem* **34** 330–336.

Trowell H C 1986 Development of the dietary fibre hypothesis of diabetes mellitus. In: *Handbook of Dietary Fibre in Human Nutrition*, ed Spillar G A. CRC Press, New York, pp 345–347.

Wali Y A, Hasan Y M 1965 Qualitative chromatographic survey of the sugars prevailing in some horticulture crops. *Proc Amer Hort Soc* **87** 264–269.

Wills R B H, Lim J S K, Greenfield H 1986 Composition of Australian foods 31. Tropical and sub-tropical fruit. *Food Tech Aust* **38** 118–123.

J Sci Food Agric 1990, **51**, 193–205

Assessment of Lipid Oxidation in Indonesian Salted-dried Marine Catfish (*Arius thalassinus*)*

Gillian Smith, Mike Hole and Steven W Hanson

School of Food and Fisheries Studies, Humberside College of Higher Education, Nun's Corner, Grimsby, South Humberside DN34 5BQ, UK

(Received 20 January 1989; revised version received 28 June 1989; accepted 25 July 1989)

ABSTRACT

Analysis of Indonesian salted-dried catfish (Arius thalassinus *Ruppell*) *during processing and storage indicates that although peroxide, thiobarbituric acid and anisidine values give an indication of the onset of lipid oxidation, the values become negligible in the consumed product. Alternative methods have been developed and the significant, steady increases found in the levels of acetic acid soluble colour and fluorescence indicate that these parameters are realistic indicators of the tertiary products formed by interactions of carbonyl compounds with amino-type compounds. These increases are complemented by a fall in the level of free amino acids during storage of the product. A 30% loss of polyunsaturated fatty acids was found during salting, but no subsequent loss was found during drying and storage.*

Key words: Lipid oxidation, salted-dried fish, fluorescence, browning, marine catfish, *Arius thalassinus*.

INTRODUCTION

It was pointed out in an FAO review on the assessment and prevention of cured fish losses (FAO 1981) that harmful compounds such as lipid oxidation products might be present in cured fish but that little information was available with respect to their levels and whether they may be causing health, especially nutritional, problems.

Cured fish may be prepared by drying, smoking or salting, and combinations of these treatments. The curing process is important in the south-east Asian region

* Part of this paper was presented at the seventh session of the Indo-Pacific Fishery Commission Working Party on Fish Technology and Marketing, Bangkok, Thailand, 19–22 April 1988.

J Sci Food Agric 0022-5142/90/$03.50

where about 30% of the marine catch is utilised in this way. During the drying process, the water activity of the fish decreases to 0·70–0·75. Salted–dried fish would be expected to have a shelf life of 4 to 12 weeks at these water activities, with the main types of spoilage including mould growth, beetle infestation and rancidity development (Poulter *et al* 1982; Hanson and Esser 1985; Esser *et al* 1986).

After death, fish lipids are subject to two major changes: lipolysis (hydrolysis) and oxidation, both of which may proceed via enzymic and non-enzymic mechanisms. Hydrolysis mainly occurs through the action of lipases (Olley and Lovern 1960; Koizumi *et al* 1980).

The normal mechanism of lipid oxidation in processed flesh foods is autoxidation, but photo-oxidation and enzymic (lipoxygenase) catalysed oxidation also result in the production of hydroperoxides (Frankel 1985; Chan 1987; Chan and Coxon 1987). Further reactions of these peroxides produce aldehydes and ketones, eg malondialdehyde, together with other secondary breakdown products (Gunstone 1984; Frankel 1985; Gardner 1987; Grosch 1987).

When lipid oxidation occurs in foods such as salted-dried fish, further reactions are possible between the primary and secondary lipid oxidation products and other, mainly nitrogenous, food constituents, eg protein, amino acids and phospholipids (mainly phosphatidylethanolamine), to give tertiary products and the possibility of browning. Measurements of fluorescence, believed to be derived from interactions between carbonyl compounds and the amino group of amino acids (Chio and Tappel 1969; Kikugawa *et al* 1981), together with the determination of colour arising from further Maillard type reactions of the fluorescent Schiff base type compounds (Smith 1988), should give some indication of the extent of these tertiary reactions.

Lipid oxidation may also lead to nutritional damage, including losses of essential fatty acids, fat soluble vitamins and essential amino acids (Eriksson 1987), and the development of toxicity (Addis *et al* 1983; Ames 1983; Pearson *et al* 1983), but little is known regarding the extent of such reactions in salted-dried fish.

In this paper the suitability of standard methods for assessing the degree of lipid oxidation in salted-dried catfish (*Arius thalassinus*) during processing and storage was examined. These were peroxide value, thiobarbituric acid value, anisidine value, iodine value and polyunsaturated fatty acid (PUFA) losses using gas chromatography of individual fatty acid methyl esters (FAMEs). Alternative techniques were also developed to measure the levels of tertiary products. This was achieved by monitoring fluorescence and browning. Additionally, the changes in the levels of free fatty acids and free amino acids were determined.

EXPERIMENTAL

Materials

Fish samples

Marine catfish (*Arius thalassinus* Ruppell) were purchased unprocessed at the landing site in Cirebon, Indonesia, and then processed in Cirebon as described below.

Twelve catfish were graded according to size into four 'small' (< 1 kg), four 'medium' (~ 1 kg) and four 'large' (< 2 kg). They were then split, gutted and washed. Three fish, one of each size, were subsequently taken to be analysed as control (unprocessed) fish. The remaining fish were immersed overnight in saturated brine at ambient temperatures (25–30°C). After salting, three fish, one of each size, were removed for later analysis at Humberside College. The other fish were placed on drying trays constructed of bamboo matting, which were placed outside in the sun to dry until the fish were approximately 50% of their original weight (2–5 days). A further three fish, as above, were removed after this stage for later analysis in Grimsby. The remaining three samples were stored at ambient temperature (25–30°C) inside a wooden box in the processing premises at Cirebon for 10 weeks. This time interval coincided with the next visit to the processor after the initial salt-drying.

All samples were frozen immediately after being taken, and were sent by air to Humberside College where they were stored at −60°C until analysed. Prior to analysis, whole fish (ie flesh, skin and bones) were sliced and minced while frozen and the mince was thoroughly mixed before samples were taken.

The proximate composition (moisture, salt, lipid and protein) of the fish samples were determined according to the methods of Egan *et al* (1981).

Chemicals

All reagents and solvents were AR grade where possible, otherwise GPR grade, and obtained from May and Baker Ltd (Dagenham, Essex), BDH Ltd (Poole, Dorset) or Fisons Scientific Apparatus Ltd (Loughborough, Leics). Standard FAME mixes for peak indentification on the gas chromatograph were obtained from Alltech Associates (Carnforth, Lancs).

Methods

Standard methods

Standard methods were used for lipid extraction (Bligh and Dyer 1959, as modified by Hanson and Olley 1963) and determinations of peroxide value (Lea 1952), Wij's iodine value, expressed as g iodine (per 100 g lipid) (AOAC 1984), and free fatty acid value (Egan *et al* 1981) on the lipid. A modified procedure, involving the use of glacial acetic acid to clarify some solutions, was used for the anisidine value (Egan *et al* 1981).

TBA value

The malondialdehyde content was determined directly on a known weight of fish sample (*w* g) using a TBA distillation method (Tarladgis *et al* 1960) with the following modification (Banasihan 1985): 1·0 ml of 2 g litre^{-1} ethylene diaminetetracetic acid (EDTA) disodium salt solution and 5 drops of an antioxidant mixture (0·3 g of butylated hydroxyanisole (BHA) in 5·4 g of propylene glycol mixed with 0·3 g of butylated hydroxytoluene (BHT) in 4 g of Tween 40) were added to the acidified sample mixture in a 2-neck round-bottomed flask. The distillation was carried out under nitrogen and the flask was heated at such a rate that 50 ml of distillate was collected in the shortest possible time (20 min).

A standard curve was prepared from tetraethoxypropane (TEP) solution to give solutions containing the equivalent of 0, 0·2, 0·4, 0·6, 0·8 and 1·0 μg ml^{-1} malondialdehyde (0·3056 g of TEP was dissolved in 1 litre of distilled water, 10 ml of this was diluted to 1 litre to give the standard concentration of 0·01389 nmol litre^{-1}, 5 ml of this solution contained the equivalent of 1·0 μg ml^{-1} malondialdehyde). These standard solutions and an aliquot of the distillate were subsequently treated with TBA as detailed by Tarladgis *et al* (1960). Absorbances were measured at 532 nm and related to the concentration of malondialdehyde from the calibration curve (T μg ml^{-1}).

The TBA value was calculated as:

$$\frac{500 \times T}{7 \times w} \text{ mg malondialdehyde (kg fish)}^{-1}$$

based on a measured 70% recovery of malondialdehyde in the distillation step (Banasihan 1985).

Acetic acid soluble colour (AASC)

This was determined on the fish samples using the method developed by Eshiet (1984). Glacial acetic acid (50 ml (v ml)) was added to 0·5–1·5 g of a representative fish sample, accurately weighed (w g), in a 150-ml beaker. After stirring the mixture for 15 min using a magnetic stirrer, the contents of the beaker were filtered through Whatman GP filter paper. The absorbance (E) of the filtrate was measured at 400 nm.

$$\text{AASC} = \frac{v \times E}{w} \text{ absorbance units ml g}^{-1}$$

Fluorescence

This was determined on the fish tissue by a method adapted from Fletcher *et al* (1973). Fish tissue (about 1·5 g (w g)) was accurately weighed into a homogenising flask and 20 ml of chloroform together with 10 ml of methanol were added. After blending for 90 s in a 45°C water bath, 30 ml of distilled water was added before homogenising for a further 30 s. The homogenate was transferred to glass centrifuge tubes and centrifuged at 2000 × g for 5 min. Aliquots of the upper aqueous and lower organic layers were collected, and filtered if cloudy. A few drops of methanol were added to the organic layer if this was still cloudy after filtering. The organic layer was then exposed to a source of high intensity UV light (Camag, 254 nm) to destroy any retinol present. The excitation and emission spectra of the extracts were determined and subsequent fluorescence measurements (F) were made at an excitation wavelength of 362 nm and an emission wavelength of 440 nm with a Shimadzu spectrofluorophotometer RF-540.

To ensure linearity of fluorescence measurements with respect to the concentration of fluorescent material, the following procedure was carried out. Serial dilutions of a very highly fluorescent extract were made, the fluorescence was measured at the above wavelengths, and a graph was plotted of fluorescence against dilution. The limit of linearity was noted, and it was subsequently ensured that all

fluorescent readings fell in the linear region of the curve. This was achieved by suitable dilution of extracts.

Quinine sulphate solution (1 μg ml^{-1} in 0·1 M sulphuric acid) was used as a standard of fluorescence intensity (S). The fluorescence of the sample relative to the standard was calculated using the following formula:

$$\text{Fluorescence (ml g}^{-1}) = \frac{F \times V}{S \times w}$$

where V ml = volume of extract (diluted where necessary) giving fluorescence F.

FAMEs

A modified methylation procedure (IUPAC 1979; Salter 1988) was used to obtain FAMEs from the fish lipid. These were separated and analysed using an SP2330 packed column in a Shimadzu GLC–RIA gas chromatograph with a Shimadzu GC 1 data processor. Nitrogen was used as the carrier gas with a flow rate of 25 ml min^{-1}, initial temperature 180°C for 5 min rising to 230°C at a rate of 4°C min^{-1}. Nonadecanoic acid was used as an internal standard at a concentration of 1 mg ml^{-1} and eluted directly after peak F, Fig 2, which corresponded to a region of the profile which gave no significant peaks. The final relative fatty acid concentrations (C) were calculated per 100 mg of lipid methylated and expressed relative to 19:0 = 100.

$$C = \frac{S \times 0{\cdot}1 \times 100}{WP}$$

where S = sample peak area; P = standard peak area; and W = weight of sample (g).

Standard PUFA retention times, hydrogenation of FAMEs (Hardy *et al* 1979; Christie 1982) and comparisons with previous work with the SP2330 column for fish oil FAMEs analysis (Christie 1982; Ahmad 1985) were used for identification of peaks.

Free amino acids

The copper method of Pope and Stevens (1939) was used to determine amino nitrogen.

RESULTS

Proximate composition

The data in Table 1 show that, during drying, the moisture content decreased from approximately 740 to 440 g kg^{-1}, with accompanying apparent increases in levels of salt, lipid and protein. The final moisture content of the stored sample, at 467 g kg^{-1}, was slightly but significantly higher than the dried value, indicating an absorption of moisture during storage. This absorption would account for the apparent decrease in protein content during storage. The reason for the increase in salt over this period is unclear, but it may be due to sampling variation.

TABLE 1

Proximate analysis[a] and free amino acid (AA) content of salted-dried catfish (*Arius thalassinus*) during processing and storage (g kg^{-1})[b]

	Unprocessed		*Salted*		*Dried*		*Stored*	
Moisture	743·1	(17·5)	694·5	(12·6)	435·0	(10·8)	467·2	(7·7)
Salt	0·8	(0·1)	52·6	(1·6)	102·5	(3·1)	117·2	(2·5)
Lipid	32·4	(8·1)	37·6	(12·9)	55·0	(11·9)	54·5	(15·6)
Protein	224·0	(10·8)	216·1	(12·6)	407·0	(17·6)	361·3	(20·1)
Free amino acid nitrogen in flesh	0·176	(0·052)	1·29	(0·59)	5·27	(0·23)	2·41	(0·75)
Free amino acid in flesh	1·55	(0·45)	11·33	(5·23)	46·34	(2·02)	21·19	(6·56)
Free amino acid in protein	7·0	(2·2)	53·3	(26·3)	114·0	(8·5)	59·4	(20·2)

[a] Wet weight basis.

[b] All values are means of 9 determinations (3 × 3 fish) with standard deviations in parentheses. A conversion factor of 8·8 was used to calculate the amino acid content from amino acid nitrogen.

Changes in standard parameters

Figure 1 shows the changes in peroxide, anisidine, TBA, free fatty acid and iodine values during salting, drying and storage of catfish (*A thalassinus*).

Peroxide values increased to a maximum during the processing before falling to zero in the stored product as found by Eshiet (1984) and Jeong and Park (1983) for salted-dried mackerel (*Scomber scombrus*) mince and sardine (*Sardinops melanosticta*), respectively. The peroxide value of the fresh (unprocessed) catfish was 43 mEq kg^{-1} lipid and as such would be classified as rancid by Connell (1980). This value may have arisen after catching, since the fresh fish may be held at tropical ambient temperatures (25–30°C) before being sold for processing. Overall, it is possible to conclude that peroxide values are unreliable indicators of lipid oxidation since a low value, in isolation, may indicate either limited or extensive lipid oxidation.

The TBA and anisidine values (Fig 1) also reach maxima and then decrease in the stored products, indicating that these determinations are also unsuitable measures of advanced lipid oxidation.

The free fatty acid (FFA) results shown in Fig 1 demonstrate that extensive hydrolysis of the lipid has occurred upon salting the fish (up to 300 g kg^{-1} FFA), with a final level of 500 g kg^{-1} FFA in the stored samples, as also found by Lovern (1962).

The fresh catfish had an iodine value (IV) of 118 which decreased by 20% during processing and storage. This is consistent with loss of polyunsaturation through lipid oxidation.

Fatty acid profiles

A typical fatty acid profile for *A thalassinus* lipid is shown in Fig 2. If this is compared with profiles of Atlantic mackerel (*Scomber scombrus*; Ackman 1980),

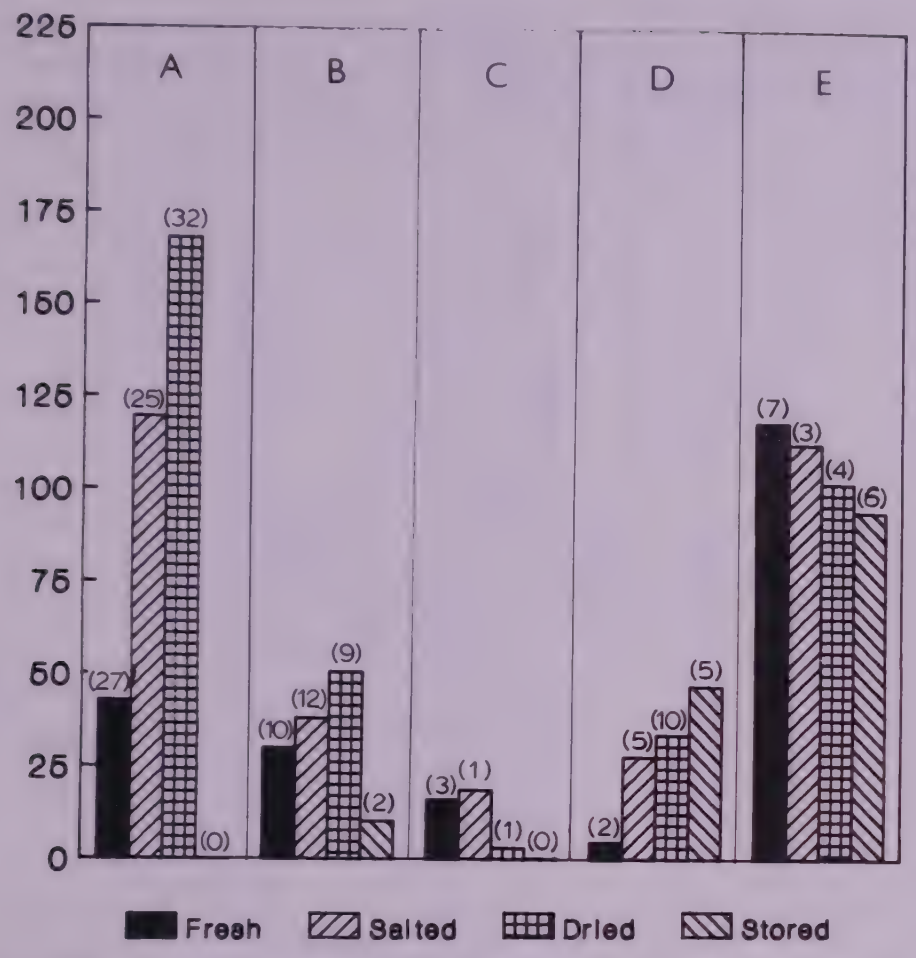


Fig 1. Lipid oxidation parameters for *Arius thalassinus* during processing. All A values are means of triplicate determinations on three fish (ie nine values), with SD in parentheses (a) Peroxide value (mEq kg^{-1} lipid); (B) anisidine value (absorbance g^{-1} lipid); (C) TBA value (mg MA kg^{-1}); (D) free fatty acid value (g oleic acid $kg^{-1} \times 0{\cdot}1$); (E) iodine value (g per 100 g lipid).

then several differences emerge, including greater saturation of the *Arius* lipid and a greater *n*-6/*n*-3 ratio. In particular 5·2% of arachidonic acid (20:4*n*-6) is present in *Arius* lipid compared with levels of $<2\%$ for *S scombrus*. These trends have been observed elsewhere in tropical marine fish (Gopakumar and Nair 1972; Gibson 1983; Fogerty *et al* 1986). Additionally, in Fig 2, no clear peak for linoleic acid is apparent, ie it is present at a concentration of $<1\%$.

To allow a comparison of the effect of processing on the fish lipid, methyl nonadecanoate was added as an internal standard and the sample peak sizes were calculated relative to this. The significance of the change in levels of fatty acids, shown in Table 2, was analysed using a Student's *t*-test. There was a significant decrease ($P<0{\cdot}05$) in the levels of each of the C20 and C22 polyunsaturates upon salting, giving an average decrease of 28·4%, but not in the case of any other fatty acid. A more unexpected change in levels of PUFA, particularly 20:4(*n*-6), 22:4(*n*-6) and 22:6(*n*-3), appeared during storage of salted-dried catfish in that these fatty acids showed an apparent increase over this stage. This may be attributable to possible sample variation. Therefore, the determination of changes in the fatty acid profile appears to be unsuitable as a measure of the extent of lipid oxidation in this product.

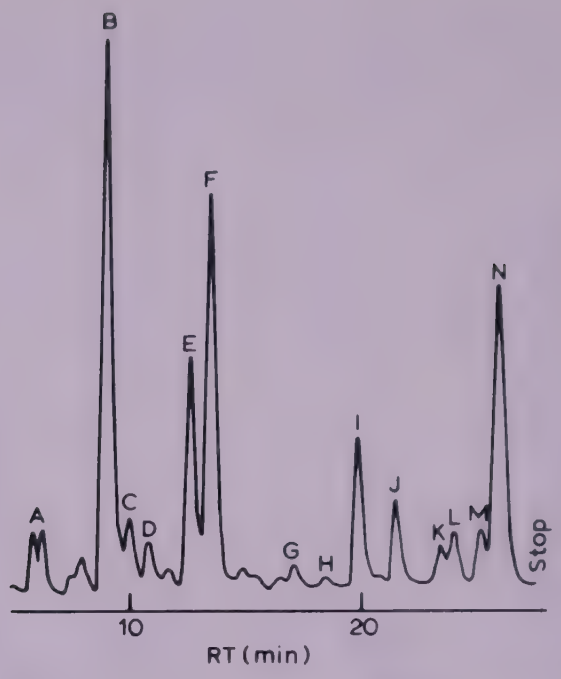


Fig 2. The fatty acid profile of fresh (unprocessed) catfish (*Arius thalassinus*) lipid

Peak	*Assignment*	*% of total*	
		Mean (*3 fish*)	*SD*($n-1$)
A	14:0	2·1	0·1
B	16:0	27·2	1·4
C	16:1(*n*-7)	3·4	0·05
D	17:0	2·5	0·2
E	18:0	10·7	0·3
F	18:1(*n*-9/*n*-7)	19·3	1·5
G	18:3(*n*-3)	1·5	0·1
H	18:4(*n*-3)	0·4	0·07
I	20:4(*n*-6)	5·2	0·5
J	20:5(*n*-3)	2·9	0·3
K	22:4(*n*-6)	1·8	0·2
L	22:5(*n*-6)	2·4	0·1
M	22:5(*n*-3)	2·1	0·25
N	22:6(*n*-3)	13·7	0·9

The changes in PUFA are reflected by changes in the calculated iodine values (also shown in Table 2) derived from a computer program input with the percentage of the component fatty acids. The trend in calculated IVs agrees with the trend for Wij's IVs during salting and drying but not during storage as the Wij's IV shows a further decrease at this stage. The Wij's IVs were consistently lower than the calculated IVs, which may be due to polymerisation of the lipid, a chemical change which is also reflected by the decrease in values of total peak area recorded after salting and drying (Table 2). Polymerised lipid will form part of the weight of lipid taken for the Wij's determination, but will not be included in the calculated values. Therefore, if the polymeric material is mostly saturated, then the Wij's IV will show

TABLE 2
The fatty acid profiles of catfish (*Arius thalassinus*) lipid at various stages of processing. Average peak areas relative to 19:0=100

Peak	*Unprocessed*	*Salted*	*Dried*	*Stored*
14:0	17·7 (1·0)	15·2 (1·4)	16·3 (1·9)	12·9 (0·9)
16:0	230·2 (12·2)	212·7 (32·8)	211·6 (26·3)	193·0 (28·3)
16:1	28·7 (0·5)	26·9 (3·4)	27·6 (1·8)	26·3 (2·8)
17:0	21·3 (2·0)	18·2 (2·9)	18·5 (0·2)	17·3 (2·4)
18:0	90·3 (2·6)	83·8 (11·6)	86·5 (9·0)	83·8 (5·8)
18:1	163·6 (13·1)	151·1 (14·5)	141·9 (19·9)	140·3 (18·7)
19:0	100·0	100·0	100·0	100·0
18:3	12·4 (0·8)	14·0 (0·6)	11·7 (0·6)	11·5 (0·4)
18:4	3·1 (0·6)	4·1 (1·0)	4·2 (0·7)	4·9 (0·9)
20:4	43·8 (4·2)	30·6 (7·1)	28·0 (3·1)	37·4 (5·3)
20:5	24·3 (2·9)	16·3 (1·1)	14·9 (2·3)	17·1 (3·8)
22:4	14·8 (2·0)	9·4 (1·4)	8·8 (1·6)	14·5 (3·9)
22:5 (*n*-6)	20·1 (1·0)	16·9 (1·9)	17·3 (1·2)	20·9 (3·9)
22:5 (*n*-3)	17·5 (2·1)	12·7 (0·4)	12·0 (2·1)	16·3 (2·6)
22:6	116·0 (7·3)	84·3 (6·4)	79·2 (10·0)	98·0 (7·0)
Unknown	42·0 (7·5)	30·0 (8·1)	28·5 (4·4)	30·0 (6·1)
Total	946 (26)	826 (55)	807 (59)	824 (54)
Calc IV	135·2 (6·7)	123·4 (8·0)	114·3 (2·6)	127·5 (7·7)
Wij's IV	118·4 (11·5)	112·6 (7·9)	101·9 (5·4)	94·3 (8·8)

All values are mean of 9 determinations (3 × 3 fish) with standard deviations in parentheses.

an overall lower degree of unsaturation per unit weight of lipid than calculated values.

Colour and fluorescence

Figure 3 shows the changes in values of acetic acid soluble colour, and fluorescence (chloroform/methanol soluble), during salting, drying and storage of catfish. The colour values remained relatively constant during salting and drying but increased during storage. Fluorescence values, however, remained low during salting but thereafter increased. The observed development of fluorescence which precedes the formation of colour is as expected (Chio and Tappel 1969; Kikugawa *et al* 1981; Pokorny 1981). The increases in colour and fluorescence suggest that these could be more reliable indicators of lipid oxidation during storage of salted-dried fish than the standard methods.

Free amino acids

Despite some large variations between the fish samples, the data in Table 1 clearly show that free amino acids accumulate (through proteolysis) during the salting and drying stages of fish processing. Additionally, there is a significant decrease in the level of free amino acid nitrogen during storage of the product. Similar losses of amino nitrogen in dried fish have been observed by Lee *et al* (1982) and Byun *et al* (1978). A 54% decrease in available lysine has also been observed during the

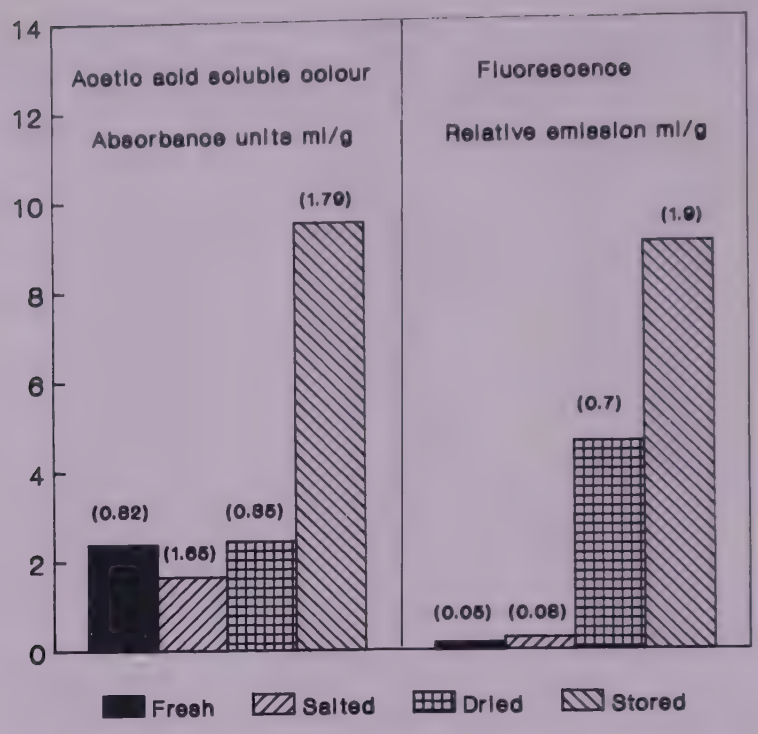


Fig 3. Changes in colour and fluorescence of *Arius thalassinus* during processing. All values are means of triplicate determinations on three fish (ie nine values) with SD in parentheses.

processing and storage of catfish (Smith 1988). These results reinforce the conclusion of Lee *et al* (1982) that available lysine and extractable nitrogenous compounds are involved in the initial stages of brown pigment formation.

DISCUSSION

Fluorescence and acetic acid soluble colour appear to be more useful indicators of lipid oxidation during processing and storage of salted-dried *Arius* than peroxide, TBA and anisidine values.

The origin of the fluorescence and colour has been investigated with model systems of aerated fish oil at 25°C. These preliminary studies indicate that the products of lipid oxidation react with amino compounds to produce fluorescence and browning (Smith 1988). No browning or fluorescence developed during oxidation of the fish oil alone at 25°C. Interactions with phospholipid resulted in chloroform/methanol soluble fluorescence while reactions with amino acids led to chloroform/methanol and aqueous soluble fluorescence. Although protein was not involved in the development of fluorescence in a protein/fish oil mixture, protein and amino acids were found to contribute to browning when water (a polar solvent) was present.

Similar reactions are likely in the salted-dried fish, as demonstrated by the reduction in the level of free amino acids which accompanies the increases in fluorescence and browning during storage of the product.

An approximately 30% loss of PUFA was apparent during the salting of catfish, but no further significant change was observed during subsequent drying and

storage of the product, despite a further decrease in Wij's IV (see the section 'Fatty acid profiles'). It must also be noted that after storage of salted-dried catfish, although zero levels of hydroperoxides and malondialdehyde were recorded, high levels of potentially oxidisable PUFA remained. The suggested antioxidant properties of 'polymeric', Maillard type lipid oxidation products (El-Khateeb and El-Zeany 1982) present in the catfish may account for this observation.

It is likely that values of fluorescence can be correlated with nutritional quality to give an indication of the potential useful shelf-life of this type of product.

ACKNOWLEDGEMENT

We are indebted to the Overseas Development Administration (ODA) for providing the funding for one of us (G Smith).

REFERENCES

Ackman R G 1980 Fish lipids. Part 1. In: *Advances in Fish Science and Technology*, ed Connell J J. Fishing News Books, Farnham, pp 86–103.

Addis P B, Csallany A S, Kindom S E 1983 Some lipid oxidation products as xenobiotics. In: *Xenobiotics in Foods and Feeds*, eds Finley J W & Schwass D E. American Chemical Society, Washington, DC, pp 85–98.

Admad N 1985 Investigation of nutritionally important fatty acids. M Phil thesis, University of Loughborough.

Ames B N 1983 Dietary carcinogens and anticarcinogens. *Science* **221** 1256–1264.

AOAC 1984 Iodine value. In: *Official Methods of Analysis* (14th edn), Association of Official Analytical Chemists, Washington, DC, p 506.

Banasihan E T 1985 Inhibition of oxidative rancidity development in minced fatty fish. PhD thesis, University of Reading.

Bligh E G, Dyer W J 1959 A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37** 911–917.

Byun D S, Song Y O, Pyeun J H 1978 Changes in available lysine and lipid oxidation products during the storage of dried sea eel (*Muraesox cinereus*). *Korean J Food Sci Technol* **10** 387.

Chan H W S 1987 The mechanism of autoxidation. In: *Autoxidation of Unsaturated Lipids*, ed Chan H W S. Academic Press, London, pp 1–16.

Chan H W S, Coxon D T 1987 Lipid hydroperoxides. In: *Autoxidation of Unsaturated Lipids*, ed Chan H W S. Academic Press, London, pp 17–50.

Chio K S, Tappel A L 1969 Synthesis and characterisation of the fluorescent products derived from malonaldehyde and amino acids. *Biochemistry* **8** 2821–2827.

Christie W W 1982 *Lipid Analysis* (2nd edn). Pergamon Press, Oxford.

Connell J J 1980 *Control of Fish Quality*. Fishing News (Books), Farnham.

Egan H, Kirk R S, Sawyer R 1981 *Pearson's Chemical Analysis of Foods* (8th edn). Churchill-Livingstone, London.

El-Khateeb S Z, El-Zeany B A 1982 Oxidised lipids – proteins browning reactions. IX. Effect of lipoinsoluble brown products on subsequent browning reactions. *Riv Ital Sost Grasse* **56** 257–259.

Eriksson C E 1987 Oxidation of lipids in food systems. In: *Autoxidation of Unsaturated Lipids*, ed Chan H W S. Academic Press, London, pp 207–231.

Eshiet I N 1984 The influence of salt on rancidity development in salted dried fish. MPhil thesis, University of Loughborough.

Esser J R, Hanson S W, Rosenthal A J, Tausin S, Harijanto 1986 *Report on the Training Programme Carried Out in East Java During October and November 1986*. Overseas Development Administration, London. Directorate General of Fisheries, Jakarta.

FAO 1981 The prevention of losses in cured fish. *FAO Fish Technology Paper, No 219*, FAO, Rome.

Fletcher B L, Dillard C J, Tappel A L 1973 Measurement of fluorescent lipid peroxidation products in biological systems and tissues. *Anal Biochem* **52** 1–9.

Fogerty A C, Evans A J, Ford G L, Kennet B H 1986 Distribution of $\omega 6$ and $\omega 3$ fatty acids in lipid classes in Australian fish. *Nutr Rep Int* **33** 777–786.

Frankel E N 1985 Chemistry of free radical and singlet oxidation of lipids. *Prog Lipid Res* **23** 197–221.

Gardner H W 1987 Reactions of hydroperoxides: products of high molecular weight. In: *Autoxidation of Unsaturated Lipids*, ed Chan H W S. Academic Press, London, pp 51–93.

Gibson R A 1983 Australian fish—an excellent source of both arachidonic and $\omega 3$ polyunsaturated fatty acids. *Lipids* **18** 743–752.

Gopakumar K, Nair M R 1972 Fatty acid composition of eight species of Indian marine fish. *J Sci Food Agric* **23** 493–752.

Grosch W 1987 Reactions of hydroperoxides: products of low molecular weight. In: *Autoxidation of Unsaturated Lipids*, ed Chan H W S. Academic Press, London, pp 95–139.

Gunstone F D 1984 Reaction of oxygen and unsaturated fatty acids. *J Amer Oil Chem Soc* **61** 441–447.

Hanson S W, Esser J R 1985 *Investigation into Post Harvest Losses of Cured Fish in South East Asia. ODA Project R3693. Report 9, Final Report*. Overseas Development Administration, London.

Hanson S W F, Olley J 1963 Application of the Bligh and Dyer method of lipid extraction to tissue homogenates. *Biochem J* **89** 101.

Hardy R, McGill A S, Gunstone F D 1979 Lipid and autoxidative changes in cold stored cod (*Gadus morhua*). *J Soc Food Agric* **30** 999–1006.

International Union of Pure and Applied Chemists (IUPAC) 1979 *Standard Methods for the Analysis of Oils, Fats and Derivatives* (6th edn). IUPAC, New York, pp 96–102.

Jeong H K, Park Y K 1983 Condition of processing and change of chemical composition on the salted dried sardine during storage. *Bull Korean Fish Soc* **16** 231.

Kikugawa K, Machida Y, Kida M, Kurechi T 1981 Studies on peroxidised lipid. III Fluorescent pigments derived from the reaction of malonaldehyde and amino acids. *Chem Pharm Bull* **29** 3003–3011.

Koizumi C, Terashima H, Wada S, Nonaka J 1980 Lipid oxidation of salted freeze-dried fish meats at different equilibrium relative humidities. *Bull Japan Soc Sci Fish* **46** 871–877.

Lea C H 1952 Methods for determining peroxide in lipids. *J Sci Food Agric* **3** 586–594.

Lee K H, Song D S, You B J, Kim M N 1982 Changes in available lysine and extractable nitrogen and extent of browning during the storage of dried fish meat. *Bull Korean Fish Soc* **15** 271–282.

Lovern J A 1962 The lipids of fish and changes occurring in them during processing and storage. In: *Fish in Nutrition*, eds Heen E & Kreizer R. Fishing News (Bookd) Ltd, London pp 86–111.

Olley J, Lovern J A 1960 Phospholipid hydrolysis in cod flesh stored at various temperatures. *J Sci Food Agric* **11** 644–652.

Pearson A M, Gray J I, Wolzak A M, Horenstein N A 1983 Safety implications of oxidised lipids in muscle foods. *Food Technol* **37** 121–129.

Pokorny J 1981 Browning from lipid protein interactions. *Prog Food Nutr Sci* **5** 421–428.

Pope C G, Stevens M F 1939 The determination of amino nitrogen using a copper method. *Biochemistry* **33** 1070–1077.

Poulter R G, Doe P E, Olley J 1982 Isohalic sorption isotherms, II. Use in prediction of storage life of dried salted fish. *J Food Technol* **17** 201–210.

Salter R M 1988 Analysis of food lipid material using capillary gas-liquid chromatography. PhD thesis, CNAA, Humberside College of Higher Education.

Smith G 1988 Lipid oxidation in south-east Asian salted-dried fish. PhD thesis, CNAA, Humberside College of Higher Education.

Tarladgis B G, Watts B M, Younathan M T, Dugan L 1960 A distillation method for the quantitative determination of malondialdehyde in rancid foods. *J Amer Oil Chem Soc* **37** 44–49.

J Sci Food Agric 1990, **51**, 207–213

Determination of Calcium and Phosphorus in Milk

T Powell Gaines,[a] Joe W West[b]

[a]Agronomy and [b]Animal Science Department, University of Georgia Coastal Plain Experiment Station, Tifton, Georgia 31793-0748, USA

and

Joseph F McAllister

The UpJohn Company, Animal Health Clinical Development, Kalamazoo, Michigan 49001, USA

(Received 5 September 1988; revised version received 12 April 1989; accepted 20 July 1989)

ABSTRACT

A fast, accurate method for determining Ca and P in milk was developed. A 1-ml samples of milk was ashed without pre-drying for 1 h and the diluted HCl extract was used for the determination of Ca by atomic absorption spectroscopy and P by colorimetry with measurement of the phosphomolybdenum blue complex at 880 nm for maximum sensitivity. Results were in good agreement with those found for 2 h ashing with or without the preliminary step for evaporating to dryness prior to ashing. Recovery ranged from 101·3 to 102·0% for Ca and 99·1 to 100·1% for P when 1 ml sample of milk was spiked with a 1-ml aliquot of a $CaHPO_4$ standard solution and analysed by the method. The method had a precision of approximately 1·0% coefficient of variation for both Ca and P in milk and in the $CaHPO_4$ standard solution.

Key words: Dairy products, elemental analysis, dry ashing, colorimetry, atomic absorption.

INTRODUCTION

Our analytical laboratory recently cooperated in a project to analyse several thousand milk samples for Ca and P from milk research studies conducted at several

J Sci Food Agric 0022-5142/90/$03.50

locations. Reviewing the literature revealed that few current methods were available for the elemental analysis of milk. Surprisingly, the Association of Official Analytical Chemists does not recommend a method for the determination of Ca or P or for elemental analysis of milk or dairy products in their Official Methods of Analysis (AOAC 1984).

Most methods for determining Ca and P in milk involve dry-ashing the sample. However, this was preceded by a preliminary heating step to evaporate the milk to dryness (Ling 1956; Murthy and Whitney 1956; Bedessem *et al* 1969; Cerbulis and Farrell 1976). The more recent methods analyse Ca using atomic absorption spectroscopy (Cerbulis and Farrell 1976) and P by colorimetry using ammonium molybdate (Bedessem *et al* 1969; Cerbulis and Farrell 1976). A recent method wet ashed the sample and determined nine elements, including Ca and P, using inductively coupled plasma emission spectroscopy (Suddendorf and Cook 1984).

The purpose of this work was to determine if a method could be developed that eliminated the time-consuming step of preliminary evaporation to dryness prior to ashing, and to refine the reported methods of determining milk P by colorimetry and milk Ca by atomic absorption spectroscopy.

EXPERIMENTAL

All reagents used were analytical grade and the water used was deionised.

Sample preparation

Samples from the morning and afternoon milking of dairy cows for a given day were combined and frozen for subsequent analysis. Samples were thawed immediately before analysis and mixed thoroughly using a vortex mixer.

Procedure

A sample (1·0 ml) of milk was pipetted into a 30-ml high-form porcelain crucible and ashed at 550°C for 1 h. After cooling, about 10 drops of deionised water were added to the ash followed by 10 ml 3 M HCl to dissolve it. The sample was placed on a hot plate and heated to a gentle boil, then filtered through Whatman No 1 filter paper (prewashed with 3 M HCl) into a 100-ml volumetric flask. The crucible was rinsed into the filter with 10 ml 3 M HCl. Once filtration was complete, the filter was half-filled with deionised water and, after the second filtration was completed, the volumetric flask was filled to volume with deionised water. Samples were shaken thoroughly and analysed for Ca and P as described.

Reagents for Ca analysis

(a) Lanthanum oxide solution (2500 μg La ml^{-1}): 29·32 g La_2O_3 slowly dissolved in 500 ml conc HCl and diluted to 1 litre with deionised water.

(b) Ca stock solution A (1000 μg Ca ml^{-1}): 3·395 g $CaHPO_4$ dissolved in 20 ml 3 M HCl and diluted to 1 litre with deionised water.

(c) Ca substock solution B (100 μg Ca ml^{-1}): 10 ml Ca stock solution A diluted to 100 ml with deionised water.

(d) Ca standards (0·5, 1·0 and 2·0 μg Ca ml^{-1}); 0·5, 1·0 and 2·0 ml of Ca substock solution B each diluted to 100 ml with La_2O_3 solution.

Ca analysis

A 1·0-ml aliquot of the filtrate was diluted to 10 ml with the La_2O_3 solution and samples were analysed for Ca using an atomic absorption spectrophotometer as previously reported (Gaines and Mitchell 1979). Samples were read in g litre^{-1} by locking in 0·500, 1·000 and 2·000 in the concentration mode for Ca standards 0·5, 1·0 and 2·0 μg ml^{-1}, respectively.

Reagents for P analysis

(a) Acid molybdate solution: 50·0 g $(NH_4)_6Mo_7O_{24}.4H_2O$ dissolved in 250 ml of 60°C deionised water until solution cleared. Cooled, then 1·22 g antimony potassium tartrate hemihydrate $K[Sb(H_2O)C_4H_2O_6]0{\cdot}5H_2O$ added and, after dissolved, 700 ml conc H_2SO_4 added slowly and the mixture diluted to 1 litre with deionised water. Stored in dark under refrigeration.

(b) Ascorbic acid solution: 88 g ascorbic acid litre^{-1} of deionised water. Stored in dark under refrigeration.

(c) P working solution: prepared fresh daily by adding the amounts shown below of acid molybdate solution, ascorbic acid solution, and deionised water for the approximate number of samples shown. Final volumes shown include amounts needed for 20 ml of five standards.

Number of samples	*Acid molybdate solution (ml)*	*Ascorbic acid solution (ml)*	*Deionised water (ml)*	*Final volume (ml)*
15	6	3	241	250
40	12	6	482	500
70	18	9	723	750
100	24	12	964	1000

(d) P stock solution A (1000 μg P ml^{-1}): 4·393 g $CaHPO_4$ dissolved in 20 ml 3 M HCl and diluted to 1 litre^{-1} with deionised water.

(e) P substock solution B (100 μg P ml^{-1}); 10 ml P stock solution A diluted to 100 ml with deionised water.

(f) P standards (3, 6, 9 and 12 μg P ml^{-1}): 3, 6, 9 and 12 ml of P substock solution B diluted to 100 ml with deionised water.

Phosphorus analysis

Aliquots (1·0 ml) of the filtrate, standards and deionised water were pipetted into 25-ml Erlenmeyer flasks and 9·0 ml of P working solution was added. After allowing a minimum of 20 min for maximum colour to develop, samples were analysed for P at 880 nm using a spectrophotometer equipped with a flow cell as previously reported (Gaines and Mitchell 1979). The 1 ml of deionised water plus 9 ml of P working solution served as a blank to zero the spectrophotometer. Samples were read in g

litre^{-1} by locking in 0·300, 0·600, 0·900 and 1·200 in the concentration mode for P standards 3, 6, 9 and 12 μg ml^{-1}, respectively.

RESULTS

Ashing time

Ashing 1 ml of a commercial sample of milk for 1 h at 550°C without evaporating the sample first on a hot plate gave Ca and P concentrations that were the same as the longer 2-h ashing with preliminary evaporation to dryness using a hot plate (Table 1). A 1-ml sample size was selected because our studies had shown that a larger volume had to be slowly evaporated to dryness on a hot plate to prevent loss of sample due to spattering in the muffle furnace. Spattering was not a problem using 1 ml in the 30-ml high-form crucible. Eliminating the preliminary evaporation step saved over 1 h in the sample preparation procedure.

Recovery of Ca and P in $CaHPO_4$ standard

The method described earlier (see 'Procedure') was subjected to a recovery study by spiking 1 ml of the commercial sample of milk (Table 1) with 1 ml of a $CaHPO_4$ standard solution that first was evaporated on a hot plate in the 30-ml high-form crucible. Then 1 ml of milk was added to the crucible and the treatments described in Table 2 were employed. The $CaHPO_4$ standard solution of 1·000 and 0·773 g per litre Ca and P, respectively, was prepared by dissolving 339·5 mg $CaHPO_4$ in 20 ml 3 M HCl and diluting to 100 ml with deionised water. Neither increasing the ashing time nor preliminary evaporation of the milk to dryness increased the Ca and P concentrations of the milk spiked with $CaHPO_4$ over the faster procedure of ashing for 1 h at 550°C with no preliminary evaporation to dryness, as there was no difference in the four treatments (Table 2). Recovery ranged from 101·3 to 102·0% and 99·1 to 100·1% for Ca and P, respectively, for the four experimental sample preparation treatments.

TABLE 1
Hot plate and ashing time effects on Ca and P determination of milk samples

Sample	*Treatment*		*Concentration*	
	Hot plate heated	*550°C Ashing time (h)*	*Ca*[a]	*P*[a]
			(*g litre*$^{-1}$)	
Milk	No	1	1·027	0·993
Milk	Yes	1	1·035	1·004
Milk	No	2	1·026	1·003
Milk	Yes	2	1·034	0·998
LSD 0·05			0·013 (NS)	0·015 (NS)
% CV			1·4	1·6

[a] Mean of 10 replicate analyses.
CV = Coefficient of variation.

TABLE 2

Hot plate and ashing time effects on Ca and P recovery of milk sample spiked with $CaHPO_4$ standard[a]

Sample	*Treatment*		*Known value*[b]		*Amount found*[c]		*Recovery*	
	Hot plate heated	*500°C Ashing time (h)*	*Ca*	*P*	*Ca*	*P*	*Ca*	*P*
			(*g litre*$^{-1}$)		(*g litre*$^{-1}$)		(%)	
Milk + $CaHPO_4$	No	1	2·030	1·773	2·071	1·769	102·0	99·8
Milk + $CaHPO_4$	Yes	1	2·030	1·773	2·066	1·775	101·8	100·1
Milk + $CaHPO_4$	No	2	2·030	1·773	2·067	1·757	101·8	99·1
Milk + $CaHPO_4$	Yes	2	2·030	1·773	2·056	1·760	101·3	99·3
LSD 0·05							2·4 (NS)	2·2 (NS)
% CV							1·3	1·6

[a] Based on evaporating 1 ml of a $CaHPO_4$ standard solution on a hot plate then adding and ashing 1 ml of milk to the crucible as described in treatments.

[b] Known Ca value: 2·030 g Ca litre^{-1} = 1·030 g Ca litre^{-1} milk value (Table 1 Ca mean) plus 1·000 g Ca litre^{-1} $CaHPO_4$ standard solution.

Known P value: 1·773 g P litre^{-1} = 1·000 g P litre^{-1} milk value (Table 1 P mean) plus 0·773 g P litre^{-1} $CaHPO_4$ standard solution.

[c] Mean of 10 replicate analyses.

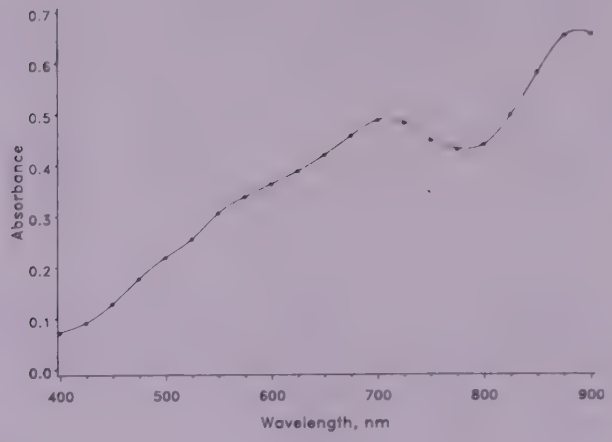


Fig 1. Phosphomolybdenum absorbance curve for 10 μg P ml^{-1} solution.

TABLE 3
Precision of Ca and P method in milk sample and $CaHPO_4$ standard

Sample	*Concentration*					
	Ca[a]			*P*[a]		
	(*g litre*$^{-1}$)	*SD*	*CV* (%)	(*g litre*$^{-1}$)	*SD*	*CV* (%)
Milk sample	1·027	0·011	1·1	0·993	0·010	1·0
$CaHPO_4$ standard	1·021	0·008	0·8	0·772	0·009	1·2

[a] Mean of 10 consecutive analyses.
SD = standard deviation.
CV = coefficient of variation.

P colorimetry

The colorimetry described previously for determining P is a modification of the Murphy and Riley (1962) method for determining P in water. This method is used extensively for determining P in soil extracts and plant tissue (Gaines and Mitchell 1979; Anon 1980). The phosphomolybdenum blue colour complex was measured at 650 nm in previously reported milk phosphorus methods (Bedessem *et al* 1969; Cerbulis and Farrell 1976); however, the absorption spectrum for phosphomolybdenum for a 10-μg P ml^{-1} solution showed that maximum absorbance and sensitivity is at approximately 880 nm (Fig 1). The wavelength effect on measuring P at 650 vs 880 nm for the P standards showed much greater absorbance and sensitivity for phosphomolybdenum measured at 880 than at 650 nm (A = 0·139, 0·277, 0·420 and 0·549 at 650 nm vs A = 0·212, 0·420, 0·634 and 0·836 at 880 nm for P standards 3, 6, 9 and 12 μg ml^{-1}, respectively).

Precision of method

The precision of the method was determined on 10 consecutive analyses of the milk sample and $CaHPO_4$ standard solution (Table 3). The standard deviation (SD) and coefficient of variation (CV) of the method for both Ca and P were small for the milk sample and the $CaHPO_4$ standard, showing that the method has good precision.

DISCUSSION

During the past year, our laboratory has used the described method to determine Ca and P concentrations in more than 4000 experimental milk samples from several milk research studies conducted at various locations. To facilitate the analyses, samples could be ashed overnight using a furnace equipped with a timer that will turn off the furnace after samples have been ashed. To ensure the accuracy of a set of analyses, a 1-ml aliquot of the $CaHPO_4$ standard solution described in Table 2 was routinely run with the milk samples as an external standard. The results reported show that the method is accurate, precise, fast and simple. One technician can make up to 100 Ca and P determinations a day using this method.

ACKNOWLEDGEMENTS

The authors are grateful to the UpJohn Company for their financial contributions to this study. Also the excellent technical assistance of Faye Childers and Betty Tyler for P analysis and Walter Harvey for Ca analysis is gratefully acknowledged.

REFERENCES

Anon 1980 *Handbook on Reference Methods for Soil Testing*. Council on Soil Testing and Plant Analysis, University of Georgia, Athens, GA.

AOAC 1984 *Official Methods of Analysis* (14th edn). Association of Official Analytical Chemists, Washington, DC.

Bedessem R V, Alioto P, Moubry R J 1969 Dry ash spectrophotometry method for the determination of phosphorus in milk. *J Assoc Offic Anal Chem* **52** 917–920.

Cerbulis J, Farrell Jr H M 1976 Composition of the milks of dairy cattle. II. Ash, calcium, magnesium, and phosphorus. *J Dairy Sci* **9** 589–593.

Gaines T P, Mitchell G A 1979 Chemical methods for soil and plant analysis. *University of Georgia Coastal Plain Exp Stn Agron Handbook 1*.

Ling E R 1956 *A Textbook of Dairy Chemistry* (3rd edn), Vol II, Chapman & Hall, London, p 86.

Murphy J, Riley J P 1962 A modified single solution method for the determination of phosphate in natural waters. *Anal Chim Acta* **27** 31–36.

Murthy G K, Whitney R M 1956 A plan for the rapid determination of the major cations in milk. *J Dairy Sci* **50** 364–373.

Suddendorf R F, Cook K K 1984 Inductively coupled plasma emission spectroscopic determination of nine elements in infant formula: collaborative study. *J Assoc Offic Anal Chem* **67** 985–992.

J Sci Food Agric 1990, **51**, 215–221

Identification of Phenolic Compounds in Edible Aroids

Tom Agbor-Egbe and June E Rickard

Overseas Development Natural Resources Institute, Central Avenue, Chatham Maritime, Chatham, Kent ME4 4TB, UK

(Received 22 May 1989; accepted 20 July 1989)

ABSTRACT

The objective of these experiments was to study the phenolic compounds present in the cormels of the edible aroids, Colocasia *spp and* Xanthosoma *spp. This investigation shows the existence of a range of phenolic compounds which includes gallic acid, chlorogenic acid, (+)-catechin, (−)-epicatechin and (−)-epigallocatechin and the possible presence of proanthocyanidins and flavonols. Aqueous acetone extracts were examined by thin layer chromatography and high performance liquid chromatography for their phenolic constituents.*

Key words: Colocasis spp, *Xanthosoma* spp, phenolic compounds, TLC, HPLC, aroids.

INTRODUCTION

Phenolic compounds found in food crops are receiving scientific attention as a result of their influence on the nutritional and sensory qualities of foods. Some of the adverse changes that occur during post-harvest storage and processing of tropical root and tuber crops have been associated with the reactions of phenolic substances (Sanchez-Nieva 1977; Ozo 1982; Rickard 1985). The corms/cormels of certain cultivars of edible aroids show enzymic browning reactions when cut and exposed to the air. This can occur with corm/cormels in the fresh state, after storage or when physically damaged. Browning in *Colocasia* spp and *Xanthosoma* spp has been attributed to the oxidation of unidentified phenolic compounds under the influence of a phenolase to produce melanins. The degree of browning was found to vary within cultivars and in different parts of a single corm or cormel (Sanchez-Nieve 1977). Chilling injury at 4°C has also been reported to cause the development of

J Sci Food Agric 0022-5142/90/$03.50

internal browning associated with physical changes in tannins in the parenchyma cells of *Colocasia* spp corms (Rhee and Iwata 1982).

The major phenols present in *C esculenta* var *esculenta* corms from Hawaii have been identified as the anthocyanidins, pelargonidin 3-glucoside, cyanidin 3-rhamnoside and cyanidin 3-glucoside (Chan *et al* 1977). In a study of *Colocasia* spp corms in Dominica using cytochemical techniques, proanthocyanidins were found to be mainly concentrated at the apical and distal ends of corms (Rickard J E unpublished).

The phenolic compounds present in the cormels of *C esculenta* var *antiquorum* and *Xanthosoma sagittifolium* have not been reported. These two species are important edible aroids in the tropical and sub-tropical regions of the world. They are a major source of food and feed. In the present study, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) have been employed to investigate the phenolic composition of these edible aroids.

EXPERIMENTAL

Materials

Fresh cormels of *Xanthosoma sagittifolium* and *Colocasia esculenta* var *antiquorum* cultivars were collected from multiplication plots belonging to the Institute of Agronomic Research, Ekona, Cameroon. Within 3 days of harvest, the cormels were peeled, washed and cut into slices which were rapidly frozen in liquid nitrogen. The samples were freeze dried for 2 days using a Chemlab bench dried and air freighted to London. In London the dried cormels were ground in a Moulinex kitchen grinder, sieved (250 μm) and extracted within 24 h of grinding.

Extraction of phenolic compounds

Freeze dried, ground samples (2 g) were homogenised with cold acetone:water (85:15 v; 25 ml) in a Waring blender at a low speed for 5 min. The slurries were filtered through glass microfibre filter papers (Whatman GF/A). The residues were reblended twice with two aliquots (10 ml each) of cold acetone:water (80:20 v) and the extracts were filtered as above. The three acetone filtrates were pooled and concentrated to approximately 10 ml in a rotary evaporator at 40 C. The concentrated extracts were washed with light petroleum (BP 60–80 C; 20 ml) to remove fatty materials. The light petroleum was decanted and the residue was removed from the extracts by evaporation under reduced pressure using the rotary evaporator at 40°C.

Chromatography of aqueous extracts

Thin-layer chromatography

Concentrated fat-free extracts of samples (10 μl) were applied to 20 × 20 cm pre-coated silica gel TLC plates using capillary tubes (Microcaps, Drummond Scientific Co, Broomall, PA, USA) and dried with a stream of nitrogen. Phenolic compounds were separated using the top layer of butan-1-ol:acetic acid:water, 4:1:5 v (BAW). After developing, the plates were immediately dried in air and observed under long-

wave ultraviolet light before and after fuming with ammonia and/or spraying with one of the following reagents.

(a) A freshly prepared solution containing vanillin (1 g) in 11 M HCl (100 ml) was used to detect flavan-3-ols, including condensed tannins, which produce a pink to orange–red coloration (Harborne 1984).
(b) Folin–Ciocalteu reagent was used as a general reagent for phenolic compounds. Phenols with catechol or hydroquinone nuclei appear blue immediately after spraying (Harborne 1984).
(c) Freshly prepared nitrous acid reagent containing equal volumes of 10% sodium nitrite, 10% acetic acid and a few drops of 2 M NaOH. Phenolic compounds produce coloured end products (Mace 1963).
(d) Diazotised *p*-nitroaniline was freshly prepared by mixing 5 mg ml^{-1} *p*-nitroaniline (2 ml), 200 mg ml^{-1} sodium acetate (8 ml) and 5 drops of 50 mg ml^{-1} sodium nitrite. This reagent produces coloured end-products with a number of phenols (Ribéreau-Gayon 1972).
(e) A freshly prepared solution of ferric reagent containing equal volumes of aqueous ferric chloride (10 mg ml^{-1}) and aqueous potassium ferricyanide (10 mg ml^{-1}) produce a blue coloration with phenolic compounds (Ribéreau-Gayon 1972).

Sprayed plates were left in an oven at 50°C for 30 min, observed and then fumed with ammonia. R_f values of the different chromatographic bands were noted. The same experimental procedure was carried out on a wide range of purified commercially available phenolic compounds alongside examples of acetone extracts.

High performance liquid chromatography

The HPLC system consisted of a Spectra Physics SP8800 ternary pump (Spectra Physics, Hemel Hempstead, Herts) linked in series to a Hewlett-Packard photodiode-array detector, Model HP1040M, which was interfaced to a Hewlett-Packard Chemstation Series 300 computer (Model 79994A). A Rheodyne 7125 injector (Spectral Physics) with a 10-μl sample loop was connected to the solvent delivery system. Separation of phenolic compounds was achieved on a μ-Bondapak (5 μm) C_{18} column (3·9 mm id × 30 cm) connected in series to a Guard-Pak precolumn module. The phenolic compounds were separated by gradient elution using 20 ml $litre^{-1}$ aqueous acetic acid (solvent A) and 100% acetonitrile (solvent B). A linear gradient from 9% (A) to 31% was achieved over a period of 50 min using a flow rate of 1·5 ml min^{-1}. Solvents A and B had been filtered through a 0·5-μm and a 0·22-μm Millipore filter, respectively. Detection was performed simultaneously at 280, 350 and 525 nm (bandwidth 4 nm) with spectrum scan from 220 to 600 nm.

Sample preparation. Phenolic compounds were extracted from freeze dried samples as already described. Sample extracts (5 ml) were injected into Sep Pak C_{18} cartridges (Millipore UK Ltd, Harrow, Middlesex), previously conditioned with acetonitrile (2 ml) and water (2 ml), to remove non-polar compounds. Samples were eluted from the cartridges with 5 ml acetonitrile:methanol:water (40:40:20 v).

Peak confirmation. Chromatographic peaks were identified by two procedures:

(a) characterisation of peaks by comparison with known absorbance wavelengths of phenolic compounds (280, 350 and 525 nm);
(b) comparison of retention times of sample chromatographic peaks with those of standard phenolic compounds, obtained using the same HPLC operating conditions.

RESULTS AND DISCUSSION

TLC

When sprayed with Folin–Ciocalteu reagent, eight blue bands appeared on the plate, indicating the presence of phenolic compounds. All the bands were colourless in visible light. Three bands fluoresced under ultraviolet light and had R_f values of 0·65 (blue), 0·62 (shell blue) and 0·44 (dull blue). Two of the fluorescent bands (R_f 0·65; 0·44) remained unchanged when fumed with ammonia vapour, and the third band (R_f 0·62) turned green indicating the presence of a caffeic acid ester. The fluorescent colour observed before and after ammonia vapour treatment of chromatograms and the lack of colour in visible light are characteristic of phenols which are either coumarin or cinnamic acid derivatives (Ribéreau-Gayon 1972). All the bands, except those with R_f values 0·65 (no colour reaction) and 0·62 (yellow), gave red–pink colours when sprayed with vanillin-HCl reagent, indicating the presence of phloroglucinol or resorcinol structures (Ribéreau-Gayon 1972). The R_f values and colour responses to spray reagents of the major phenolic bands chromatographed in BAW from acetone extracts are summarised in Table 1.

Six compounds were identified by their R_f and colour reactions by comparison with commercially available standards. The compounds identified were phloroglucinol, chlorogenic acid and t-cinnamic acid (cinnamic acids), and (+)-catechin; (−)-epicatechin; (−)-epigallocatechin (flavan-3-ols). A group of bands (R_f, 0·37, 0·31 and 0·25) with similar colour responses to all spray reagents were tentatively identified as proanthocyanidins by comparison of the results obtained with published reference values (Ribéreau-Gayon 1972; Markham 1982; Harborne 1984; Mueller-Harvey *et al* 1987). The identity of these bands could not be substantiated because of the lack of suitable standards.

High performance liquid chromatography

Fifteen peaks were identified by their absorption characteristics as phenolic compounds. A typical chromatogram obtained from sample extracts at 280 nm is shown in Fig 1. Absorption data collected during separation of sample extracts using the diode-array detector (spectrum scan 220–600 nm) showed that the phenolic compounds present had very low absorption at wavelengths greater than 375 nm indicating there were no anthocyanidins present. These compounds have maximum absorbance at 525 nm. The retention times and ultraviolet spectral characteristics of commercially available phenolic compounds (Table 2), when compared with the chromatographic peaks recorded for sample extracts,

TABLE 1

Characteristic colour responses of sample acetone fractions separated by thin layer chromatography (TLC) towards spray reagents

R_f[a] (BAW)[b]	Colour under UV light		Colour responses					Tentative identity
	Normal	After fuming with ammonia	Vanillin-HCl	Nitrous acid	Ferric chloride	Folin Ciocalteu	DPNA[c]	
0·95	—	—	Pink	Dull brown	Black	Light blue	Orange	(+)-Catechin
0·92	—	—	Pink	Yellow brown	Mauve	Blue	Orange	(−)-Epicatechin
0·81	—	—	Pink	Yellow brown	Mauve	Blue	Orange	(−)-Epigallocatechin
0·65	Blue	Blue	—	—	Yellow	—	—	t-Cinnamic acid
0·62	Shell blue	Green	Yellow	Yellow brown	Green	Blue	Purple	Chlorogenic acid
0·44	Dull blue	Dull blue	Red	Green	Brown	Blue	Orange	Phloroglucinol
0·37	—	—	Red brown	Yellow brown	Grey	Dark blue	Orange	Proanthocyanidins
0·31	—	—	Pink	Yellow brown	Grey	Dark blue	Orange	Proanthocyanidins
0·25	—	—	Light pink	Yellow brown	Grey	Dark blue	Orange	Proanthocyanidins

[a] Mean of 8 plates per sample.
[b] BAW = butanol:acetic acid:water, 4:1:5 v, top layer.
[c] DPNA = diazotised *p*-nitroaniline.

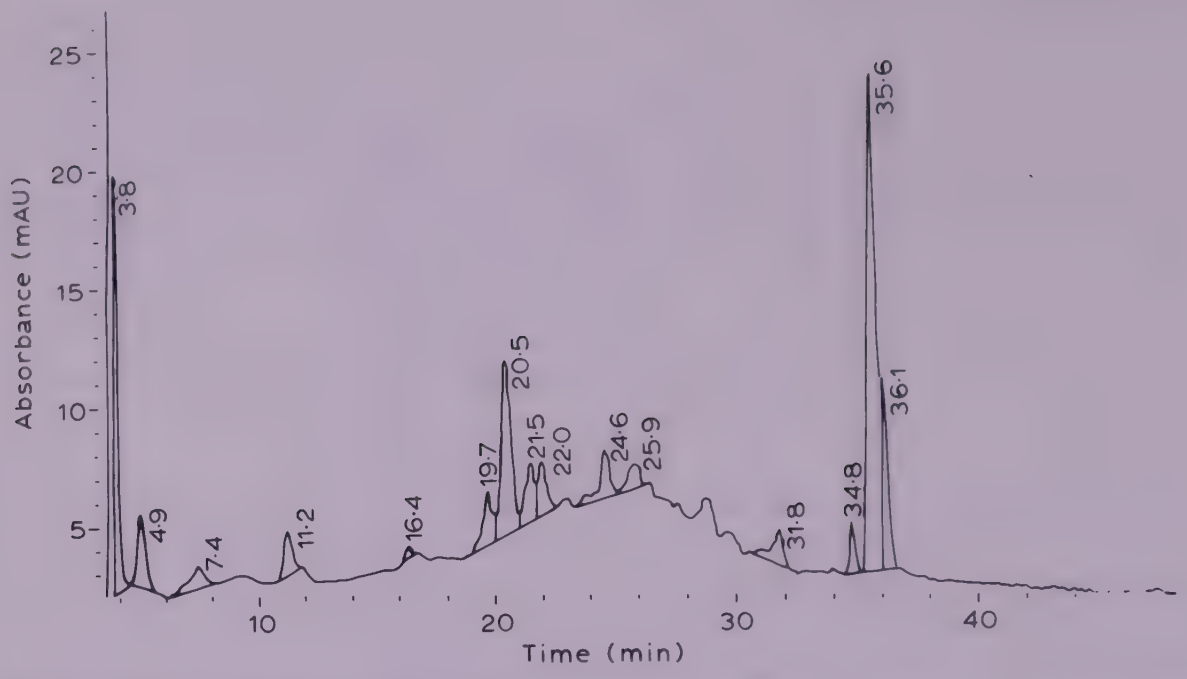


Fig 1. Typical chromatogram at 280 nm of sample phenolic compounds. Column: μ-Bondapak C_{18} (id 3·9 mm × 300 mm; mobile phase: 20 ml acetic acid per litre: acetronile; gradient elution from 9 % to 31 % acetic acid, flow rate 1·5 cm^3 min^{-1}.

TABLE 2
Retention times and ultraviolet absorption maxima of commercially available phenolic compounds

Compound	*Retention time (min)*	*Wavelength at maximum absorption (nm)*
Gallic acid	3·8	272
Phloroglucinol	4·1	268
Chlorogenic acid	4·9	244, 300sh, 328
(+)-Catechin	7·4	240, 280
(−)-Epigallocatechin	11·2	244, 290
(−)-Epicatechin	16·4	237, 280
t-Cinnamic acid	17·2	276, 320

sh, Shoulder.
Ultraviolet absorption maxima obtained in 20 ml acetic acid per litre: acetonitrile, gradient elution.

substantiated some of the identifications made using TLC. The chromatographic peaks obtained at 280 nm (Fig 1) with retention times 3·8, 4·9, 7·4, 11·2 and 16·4 min were determined as corresponding to gallic acid, chlorogenic acid, (+)-catechin, (−)-epigallocatechin and (−)-epicatechin, respectively. However, the presence in the samples of phloroglucinol and t-cinnamic acid which had been indicated using TLC were not substantiated using HPLC.

Chromatograms were also obtained at 350 nm. Peaks with retention times 31·8 and 34·8 min at 280 nm were also found to absorb at 350 nm, the stronger absorption occurring at 280 nm. It has been reported that the principal ultraviolet absorption maxima for flavonols are at 270–280 nm and 310–350 nm (Markham 1982; Harborne 1984). It is therefore possible that these two chromatographic

peaks (31·8 and 34·8 min) correspond to flavonol or their glycosides. The sample acetone extracts were not hydrolysed to test if there was liberation of aglycones. All the peaks obtained around 19–25 min, as shown in Fig 1, absorbed only at 280 nm. Procyanidins have been reported to absorb only at 280 nm with lower retention times than most flavonol and flavonol glycosides (Mueller-Harvey *et al* 1987; Oleszek *et al* 1988). It is possible that some of the peaks obtained at 19–26 min could be attributed to procyanidins, the presence of which was indicated by the TLC results. Confirmation of the identity of the remaining chromatographic peaks was not possible due to lack of standard phenolic compounds. The HPLC chromatograms of all the cultivars examined were very similar to that presented in Fig 1.

This investigation shows the existence of a wide range of phenolic compounds in edible aroid species. These compounds are gallic acid, chlorogenic acid, (+)-catechin, (−)-epicatechin and (−)-epigallocatechin; proanthocyanidins and flavonols were also possible present. Similar phenolic compounds to those reported in these experiments have been found in other root and tuber crops. Chlorogenic acid isomers have been identified in sweet potato (Walter *et al* 1979), caffeic acid, chlorogenic acid, (+)-catechins and proanthocyanidins have been detected in yams (Martin and Ruberte 1976; Ozo 1982) and chlorogenic acid has been identified in potato (Malmberg and Theander 1985). A range of phenolic compounds have been identified in cassava roots, which included scopoletin, catechins and condensed tannins (Rickard 1985).

REFERENCES

Chan H T, Kao-Jao T H C, Nakayama T O M 1977 Anthocyanin composition of taro. *J Food Sci* **42** 19–21.

Harborne J B 1984 *Phytochemical Methods* (2nd edn). Chapman and Hall, London.

Mace M E 1963 Histochemical localisation of phenols in healthy and diseased banana roots. *Physiol Plant* **16** 915–925.

Malmberg A G, Theander O 1985 Determination of chlorogenic acid in potato tubers. *J Agric Food Chem* **33** 549–551.

Markham K R 1982 *Techniques of Flavonoid Identification*. Academic Press, London.

Martin F W, Ruberte R 1976 The polyphenol of *D. alata* (yam) tubers associated with oxidative browning. *J Agric Food Chem* **24** (1) 67–70.

Mueller-Harvey I, Reed J D, Hartley R D 1987 Characterisation of phenolic compounds including flavonoids and tannins, of ten Ethiopian browse species by high performance liquid chromatography. *J Sci Food Agric* **39** 1–14.

Oleszek W, Lee C Y, Jaworski A W, Price K R 1988 Identification of some phenolic compounds in apples. *J Agric Food Chem* **36** 430–432.

Ozo N O 1982 Biochemical changes in stored yams (*Dioscorea* spp.) with special reference to polyphenols and polyphenoloxidases. PhD thesis, University of Reading.

Rhee J K, Iwata M 1982 Histochemical observations on the chilling injury of taro tubers during cold storage. *J Japan Soc Hort Sci* **51** (3) 362–368.

Ribéreau-Gayon P 1972 *Plant Phenolics* (2nd edn). Oliver and Boyd, Edinburgh.

Rickard J E 1985 Physiological deterioration of cassava roots. *J Sci Food Agric* **36** 167–176.

Sanchez-Nieva F 1977 Control of browning in lye-peeled taniers. *J Agric Univ Puerto Rico* **61** 337–344.

Walter W M, Purcell A E, McCollum G K 1979 Use of high pressure liquid chromatography for analysis of sweet potato phenolics. *J Agric Food Chem* **27** (5) 938–941.

J Sci Food Agric 1990, **51**, 223–231

Effects of Some Thermal Treatments on Polyphenoloxidase and Peroxidase Activities of Banana (*Musa cavendishii*, var *enana*)

Pilar Cano, M Antonia Marín and Carmen Fúster

Instituto del Frío (CSIC), Ciudad Universitaria, 28040-Madrid, Spain

(Received 6 June 1989; accepted 20 July 1989)

ABSTRACT

Polyphenoloxidase (EC 1.14.18.1) and peroxidase (EC 1.11.1.7) activities were evaluated during cold storage of banana (Musa cavendishii, *L var* enana). *The effects of some thermal treatments (blanching of peeled bananas, microwave treatment of banana slices and storage of banana slices at −24°C) were studied. Inactivation of both enzymes by blanching was very effective (96–100%) at all maturity levels. Microwave treatment showed differing efficiency depending on banana ripeness, and produced non-enzymic darkening due to Maillard reactions mainly in green and full-yellow bananas. Freezing of fruit slices without previous thermal treatment produced different effects in polyphenoloxidase and peroxidase activities. It was concluded that immersion in boiling water of peeled bananas with 70% green/30% yellow peel colour maturity index is the optimal pretreatment for the preservation of frozen banana slices.*

Key words: Peroxidase, polyphenoloxidase, ripening, blanching, microwave, freezing, banana, *Musa cavendishii*.

INTRODUCTION

Bananas undergo rapid browning as a result of tissue disruption and exposure to oxygen during the peeling and slicing operations prior to further processing. The quantity of bananas used for processing is insignificant by comparison with the international market for fresh fruit, but about 15% of the fruit produced for export is rejected due to size, skin stains or other factors. The successful prevention of banana browning by inactivation of enzyme by addition of sodium bisulphite has

J Sci Food Agric 0022-5142/90/$03.50

been widely discussed (Guyer and Erickson 1954; Brekke *et al* 1969; Tonaki *et al* 1973; García *et al* 1974) but blanching for enzyme inactivation of whole peeled fruits or slices has not been reported.

Polyphenoloxidase (EC 1.14.18.1; PPO) and peroxidase (EC 1.11.1.7; POD) catalyse the oxidation of phenols, which produces changes in raw fruit colour. These biochemical changes during processing and storage can produce a significant loss of quality in frozen fruit. To optimise this process, it is necessary to study the effects of some mild heat treatments and the freezing of the product on enzymic activities in bananas at different maturity levels. Several authors have reported the characteristics of banana polyphenoloxidase and peroxidase (Haard and Tobin 1971; Galeazzi *et al* 1981a) but there is no literature on the biochemical aspect of fruit preprocessing.

MATERIALS AND METHODS

Plant material

Air-freight shipments of green bananas (*Musa cavendishi* L, var *enana*), produced in the Canary Islands, were obtained from a commercial source in Madrid. Undamaged fruit free from infection were selected and stored at 14°C and 85–90% relative humidity, conditions recommended by Salunke (1984).

Samples for processing and analyses

At each storage interval ten fruits were removed from 12 bunches, each banana being selected by its situation in the bunch due to the fruit colour variability during ripening. Treated and non-treated bananas were carefully sliced (thickness, 1·1 cm), selecting only the slices with homogeneous diameter. Analyses were carried out in duplicate of these ten fruits.

Thermal treatments

Thermal treatments were applied to bananas during storage: blanching in boiling water for 11 min (whole peeled fruits); microwave treatment at 650 W for 2 min (banana slices) and freezing at −24°C for 24 h (banana slices). Blanching and microwave treatments were terminated by cooling in ice water for 5 min.

Sugars

Total and reducing sugars were analysed by the method of Gaines (1973) based on a colorimetric procedure employing the potassium ferricyanide/potassium ferrocyanide oxidation–reduction reaction.

Titratable acidity and pH

Samples were homogenised with distilled water, and a weighed portion was titrated with 0·1 M NaOH to pH 8·1 using a Crison glass electrode pH meter. The results are expressed as meq kg^{-1} fresh weight. The pH was determined in the homogenised pulp before titration.

Soluble solids

The soluble solids of raw fruit were determined with an Atago refractometer (Atago, Madrid). Results are reported as °Brix at 20°C.

Total solids

The AOAC (1984) vacuum oven method was modified employing a microwave oven operating at 650 W for 25 min.

Pulp rupture force

Each peeled banana was tested on opposite sides of the fruit. A compression force was applied to the pulp using a Bellevue pressure tester (Bellevue, Madrid) with a 7·5-mm-dia cylindrical probe. The value recorded for the pulp rupture force was the maximum force required for the pulp to yield to the tip of the penetrometer head.

Pulp/skin ratio and weight loss

Pulp/skin ratio and weight loss were determined by weighing accurately ten fruits with and without skin.

Preparation of enzyme extracts

Banana pulp (20 g) was homogenised in an ice-cooled blender jar for 3–6 min with 0·2 M sodium phosphate (pH 7·0) containing 10 g litre^{-1} insoluble polyvinylpyrrolidone (PVP) and 5 g litre^{-1} Triton X-100 (Galeazzi *et al* 1981b). The homogenates were centrifuged at 4°C for 15 min and 12 000 × *g*. The active enzymes remained in the supernate.

Protein determination

Protein concentration in all preparations was determined employing the Bio-Rad reaction according to Bradford (1976).

Measurement of enzyme activity and enzyme unit

Enzyme activity of PPO was determined by measuring the rate of increase in absorbance at 420 nm and 25°C in a model Lambda 15 double beam Perkin Elmer spectrophotometer. The reaction mixture contained 2·0 ml of 0·1 M catechol solution, 1·0 ml of distilled water and 25 μl of diluted (1 + 1) or undiluted enzyme solution depending on the sample to be analysed.

Enzyme activity of POD was determined by measuring the rate of increase in absorbance at 485 nm and 25°C. The reaction mixture contained 2·7 ml of 0·2 M citrate/0·4 M phosphate buffer, pH 6·5, with 200 μl of 10 g litre^{-1} *p*-phenylenediamine as H-donor, 100 μl of 15 ml litre^{-1} hydrogen peroxide as oxidant and 25 μl of diluted or undiluted enzyme solution.

Polyacrylamide gel electrophoresis

Electrophoresis was performed on a Mighty Small II (slab gel electrophoresis unit) SE 250 assembly (Hoefer Scientific Instruments, San Francisco) at PS 500XT DC power supply 20 mA per plate with cooling to approximately 5°C. Gels were

incubated in a 10 g litre^{-1} phenylenediamine/15 ml litre^{-1} H_2O_2/0·2 M sodium phosphate buffer (pH 6·5) solution for POD and in a solution of 60 g litre^{-1} catechol/0·2 M sodium phosphate buffer (pH 6·5) for PPO.

RESULTS AND DISCUSSION

Ripening of cold-stored bananas

The main ripening trends in bananas are similar to those reported for most other tropical fruits. During ripening sugar content increased and acidity also increased slightly (Table 1). Soluble solids and total sugars increased two- to three-fold at 14 days of storage whereas titratable acidity increased two-fold at 20 days. These trends are emphasised by changes in sugar:acid ratio, which increased throughout ripening. There is a considerable water loss through transpiration after the initiation of ripening. Despite these losses, the moisture content of banana pulp increased from 727 to 735 g kg^{-1} ($P > 0·05$). This water is possibly derived from the respiratory breakdown of carbohydrates (Salunke 1984) and is due to osmotic transfer of moisture from peel to pulp resulting in changes in pulp/skin ratios from 1·14 to 1·48 in this study.

During banana ripening the extractable protein was directly related to the fruit maturity (Fig 1). There are four different stages:

(a) a slight increase in extractable protein (5%) with a simultaneous small fall in banana firmness (Table 1);
(b) a large increase (99%) in protein with a larger decrease in fruit firmness (75%);
(c) a fall in extractable protein (13%) while the firmness was continuously decreasing; and
(d) a slight increase in protein (5%) due to the balance between protein catabolism and cellular disruption with fruit senescence.

The enzyme activities of PPO and POD are shown in Fig 1. PPO and POD activities did not remain constant throughout ripening. Both showed an increase (PPO 100%, POD 31%) at the first stages of fruit ripening (from full green to 70% green/30% yellow), then diminishing slowly (PPO 32%, POD 3%) in the full yellow banana.

These changes could be explained by Palmer's (1971) conclusion that banana ripening is primarily a differentiation process involving programmed synthesis of specific enzymes required for ripening. The first stages involved both enzymes following which POD activity was maintained but PPO activity diminished until fruit senescence commenced. At this stage both enzymes began to play a primordial role in fruit metabolism which is related to the darkening of peel and fruit.

Polyacrylamide gel electrophoresis of pulp extracts separated three different species of POD (R_f 0·59; 0·62; 0·83) on polyacrylamide gel (Fig 2). The isoenzymic change observed in banana POD extracts throughout ripening could be correlated with the evolution of enzymic activity (Fig 1). When the activity was very small (first day of storage), these species were not readily revealed by the method described.

TABLE 1

Compositional and physical changes during ripening of bananas, *Musa cavendishii* var *enana*, at 14°C

Storage time (days)	*0*	*6*	*14*	*16*	*20*	*23*
Peel colour:	*Green*	*95% Green, 5% yellow*	*30% Green, 70% yellow*	*30% Green, 70% yellow*	*5% Green, 95% yellow*	*Yellow with brown areas*
Weight (g)	109·5 ± 10·0	108·6 ± 12·7	107·6 ± 12·7	107·5 ± 6·7	106·0 ± 6·3	106·2 ± 8·2
Firmness (kg)	5·0 ± 0·5	4·6 ± 0·6	1·2 ± 0·5	1·0 ± 0·3	0·6 ± 0·1	0·2 ± 0·1
Pulp:skin ratio	1·1 ± 0·1	1·1 ± 0·0	1·3 ± 0·1	1·3 ± 0·1	1·3 ± 0·1	1·5 ± 0·1
Moisture content (g kg^{-1})	767 ± 3	716 ± 5	742 ± 5	749 ± 2	723 ± 1	735 ± 3
Soluble solids (g kg^{-1})	78 ± 12	70 ± 5	184 ± 9	164 ± 8	230 ± 10	248 ± 8
Total sugars (g kg^{-1})	15·9 ± 9	42·3 ± 10	95·7 ± 7	129·9 ± 5	180·6 ± 8	202·4 ± 3
pH	5·27 ± 0·4	5·31 ± 0·2	4·60 ± 0·6	4·54 ± 0·4	4·45 ± 0·4	4·82 ± 0·2
Titrable acidity (mEq kg^{-1})	31 ± 24	28 ± 6	34 ± 12	42 ± 20	59 ± 11	62 ± 9

Values are mean of ten fruits ± standard error.

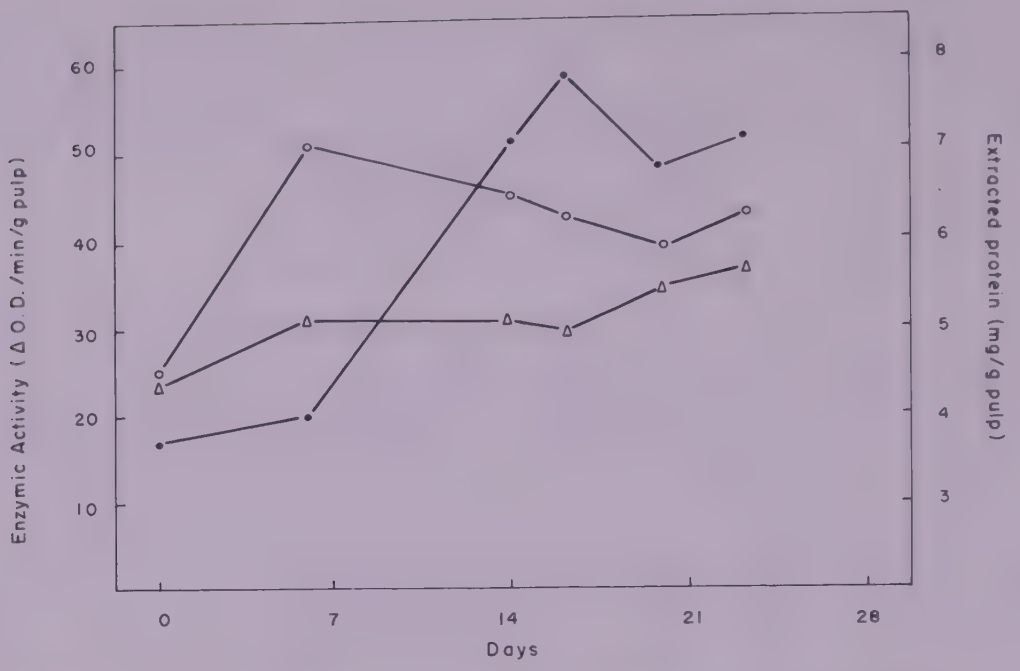


Fig 1. Changes in △ protein, ● peroxidase activity, and ○ polyphenoloxidase activity of bananas (*Musa cavendishii*, var *enana*) at 14°C.

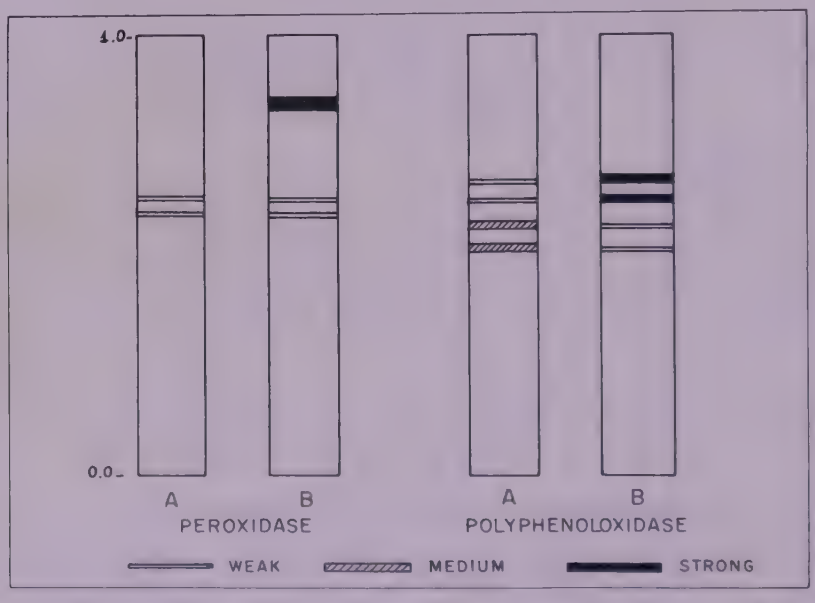


Fig 2. Polyacrylamide gel electrophoresis of the PPO and POD banana extracts. A. Dark green banana; B, full yellow banana.

The extracts of banana fruits at senescence showed a new very strong species of enzyme (R_f 0·83) which was coincident with marked increase in POD activity.

Polyphenoloxidase extracts separated into four isoenzymes with the relative mobilities: 0·51; 0·56; 0·62; 0·66. The first two species were present in full green bananas and the second two in full yellow fruit. This PPO isoenzyme pattern was similar to the banana PPO isoenzymes reported by Galeazzi *et al* (1981b) when a catechol solution was used for incubation.

Banana PPO and POD showed the species with higher relative mobility at the senescence stage (Fig 2). This evolution could be explained by assuming that these isoenzymes are the most ionic of the species and are liberated from the cellular

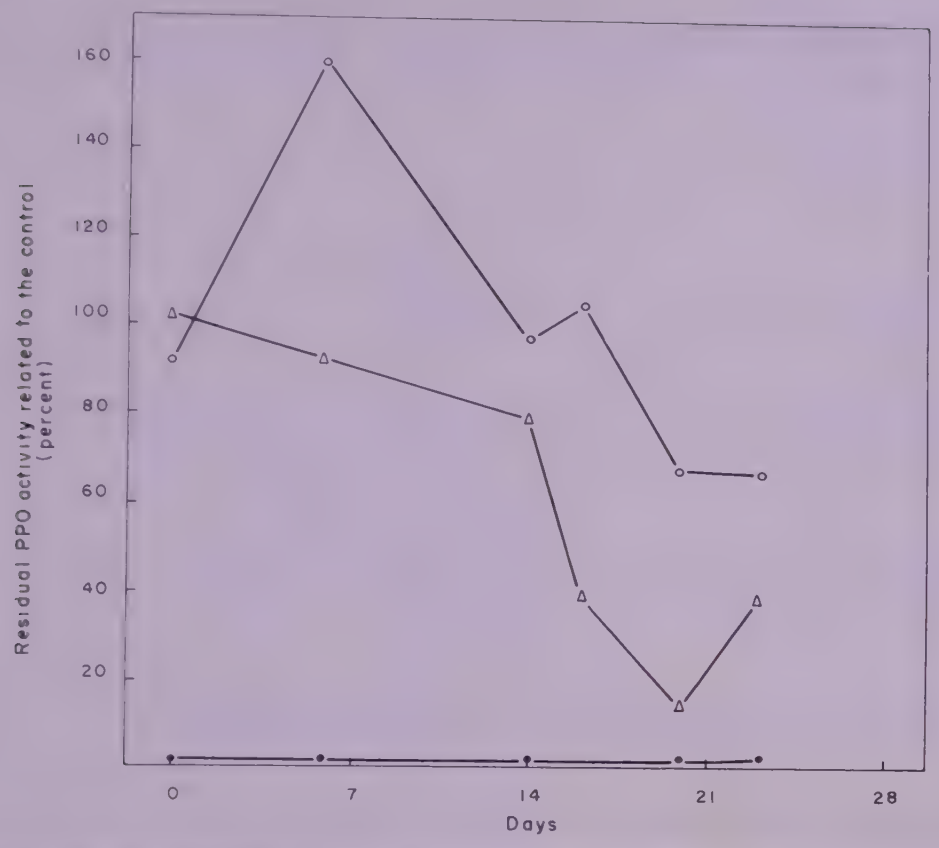


Fig 3. Effect of thermal treatments on PPO activity of banana (*Musa cavendishii*, var *enana*) stored at 14°C. ●, Blanching; △, microwave; ○, freezing.

membrane when the fruit reaches senescence and the deterioration of this membrane progressed significantly.

Effects of thermal treatments

Blanching peeled bananas in boiling water produced significant inactivation of both enzymes (96–100%). The effect was apparently not related to fruit maturity (Figs 3 and 4). The treatment conditions were drastic and produced a slight over-cooking of the final product, but this treatment is necessary to inhibit the colour deterioration of frozen banana slices if the simultaneous addition of chemical preservatives is to be avoided. This thermal treatment was employed by Garcia *et al* (1985) as a pretreatment in the processing of banana pulp stored at tropical temperatures.

The enzyme inactivation was greater for PPO than for POD. This effect was consistent with the accepted idea that POD is the more thermally stable enzyme in vegetables (Reed 1975).

Microwave treatment had different effects on PPO and POD activities depending on banana maturity level at the processing date (Figs 3 and 4). PPO was very stable when the treatment was applied to fruits stored for 14 days (0–20% PPO activity losses). From this date the inactivation increased to 85% after 20 days and to 60% when bananas at the senescence stage were processed (23 days of storage, peel yellow with brown spots).

Thermal inactivation of POD with this method in dark green fruits reached 60%, diminishing to 35% after 14 days of storage; from this stage onwards the changes were similar to those of PPO. This method produced greater inactivation of POD,

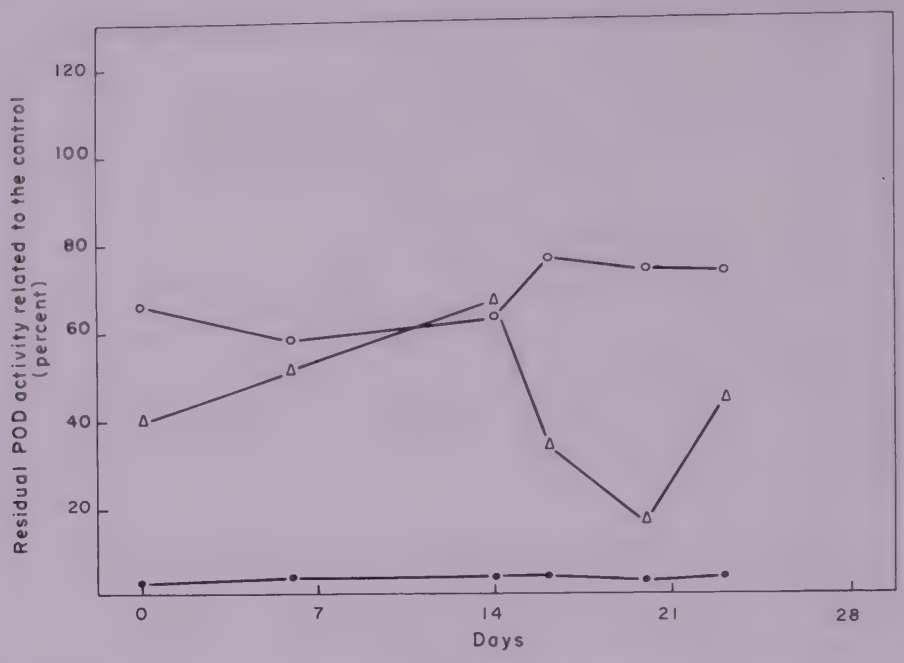


Fig 4. Effect of thermal treatments on POD activity of banana (*Musa cavendishii*, var *enana*) stored at 14°C. ●, Blanching; △, microwave; ○, freezing.

reaching smaller POD values after 20 days; thereafter POD and PPO had similar inactivation rates.

These results agree with the hypothesis reported by García *et al* (1985) which concluded that a greater starch content will reduce heat transfer rates during thermal treatments, diminishing its effectiveness.

Slices from green bananas showed a non-enzymic Maillard browning due to the high moisture content of the fruit (Table 1) when they were submitted to microwave treatment; slices from full yellow bananas also became darker. Absorption of microwave energy by vegetable tissue by virtue of the water it contains results in chemical and physical changes. Microwave heating may increase internal cell pressure leading to rupture and a loss of cell contents, oxidation and related degradative reactions. For this reason a pretreatment with microwave energy was not suitable for processing of banana slices due to these secondary non-enzymic effects on fruit colour.

The effects of freezing, without previous thermal treatments, on PPO and POD activities of banana slices differed depending on fruit maturity and the enzymic system studied (Figs 3 and 4). Peroxidase is an enzyme linked to the cellular membrane, and its solubility was affected by the freezing conditions of vegetable tissue. When the moisture content was low, more ice crystals grew and their size was dependent on the freezing method employed. In our study, it can be seen that maintaining prefixed freezing conditions, when the amount of ice crystals in the protoplasm increases, produces more mechanical damage. The POD extractability could be increasing as well as its residual activity. However, the residual activity values for PPO (Fig 3) show that there was no inactivation due to freezing in this system for the first days of storage, whereas a 34% inactivation was obtained after 20 days. The 160% activation value after 6 days could be explained by mechanical damage of cellular membranes. This increase in solubilisation of PPO was not so

important in bananas of different maturity levels. Green fruits showed little freezing damage due to high degree of tissue firmness and consequently low extractability of PPO.

REFERENCES

AOAC 1984 *Official Methods of Analysis* (14th edn). Association of Official Analytical Chemists, Washington, DC.

Bradford H 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72** 248–254.

Brekke J E, Tonaki K I, Cavaletto C G, Frank H A 1969 Stable banana puree for long-term refrigerated storage. *J Sci Food Agric* **20** 376–378.

Gaines T W 1973 Automated determination of reducing sugars, total sugars, and starch in plant tissue from one weighted sample. *J Assoc Offic Anal Chem* **56** 1419–1424.

Galeazzi M A M, Sgarbieri V C, Constantinides S M 1981a Isolation, purification and physicochemical characterization of polyphenoloxidases (PPO) from a dwarf variety of banana (*Musa cavendishii*, L.). *J Food Sci* **36** 854–857.

Galeazzi A A M, Sgarbieri V C 1981b Substrate specificity and inhibition of polyphenoloxidase (PPO) from a dwarf variety of banana (*Musa cavendishii*, L.). *J Food Sci* **46** 1404–1406.

Garcia R, Menchu J F, Rolz C 1974 Tropical fruit drying, a comparative study. *Proc IVth Int Congr Food Sci Technol* **4** 32–40.

Garcia R, Arriola M C, Porres E, Rolz C 1985 Process for banana puree preservation at rural level. *Lebensm Wiss Technol* **18** 323–327.

Guyer R S, Erickson F B 1954 Canning of acidified banana puree. *Food Technol* **8** 165–167.

Haard N F, Tobin C L 1971 Patterns of soluble peroxidase in ripening banana fruit. *J Food Sci* **36** 854–857.

Palmer J K 1971 Banana. In: *Biochemistry of Fruits and their Products*, Vol 2, ed Hulme A C. Academic Press, London, pp 65–92.

Reed G 1975 Oxidoreductases. In: *Enzymes in Food Processing*, Academic Press, New York.

Salunke D K 1984 Banana and plantain. In: *Postharvest Biotechnology of Fruits*, Vol 1, eds Salunke D K & Desai B B. CRC Press, Boca Raton, Fla, pp 43–57.

Tonaki K I, Brekke J E, Frank H A, Cavaletto C G 1973 Banana puree processing. *Research Report 202*, Hawaii Agric Exp Stn, College of Tropical Agriculture, University of Hawaii.

J Sci Food Agric 1990, **51**, 233–246

Energy Values of Lactitol and Lactulose as Determined with Miniature Pigs and Growing Rats

Susan P Bird,* David Hewitt‡ and Michael I Gurr§

AFRC Institute of Food Research, Reading Laboratory, Shinfield, Reading RG2 9AT, UK

(Received 30 December 1987; revised version received 16 May 1989; accepted 5 July 1989)

ABSTRACT

Digestible and metabolisable energy values were determined by the metabolic balance method. The mean (and SEM) metabolisable energy values for lactitol were 11·8 (0·8) and 9·8 (1·5) kJ g^{-1}, respectively, for the laboratory rat and the miniature pig. For lactulose, metabolisable energy values were 8·4 (1·0) and 9·0 (0·8) kJ g^{-1}, respectively, for the rat and pig. Digestible energy values were little different from these metabolisable energy values indicating that the efficiency of utilisation of the digestion products absorbed may be high.

Key words: Lactulose, lactitol, metabolisable energy, digestible energy, laboratory rat, miniature pig.

INTRODUCTION

This paper describes the determination of energy values by the energy balance procedure of lactitol (4-O-β-D-galactopyranosyl-D-sorbitol) and lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose), both derivatives of lactose. It is assumed that in most mammals these carbohydrates are hydrolysed only very slowly if at all (Dahlqvist and Gryboski 1965; Nilsson and Jägerstad 1987). It has been proposed that lactitol could be used in the manufacture of 'low calorie' foods (van Velthuijsen 1979), and indeed it was recently approved for food use in the UK. However, there is

* To whom correspondence should be addressed at: Milk Marketing Board, Thames Ditton, Surrey KT7 0EL, UK.
‡ Present address: Department of Applied Statistics, University of Reading, Reading, UK.
§ Present address: Milk Marketing Board, Thames Ditton, Surrey KT7 0EL, UK.

J Sci Food Agric 0022-5142/90/$03.50

little published information on its contribution to energy value. Lactulose has found medical applications as a laxative (Mayerhofer and Petuely 1959) and is used in the treatment of portal systemic encephalopathy (Bircher *et al* 1966). It may be prescribed in considerable daily quantity (20 to 100 g or more), but the contribution of this sugar to the energy metabolism of the patient has been given little consideration and no energy values are available.

EXPERIMENTAL

Materials

The lactitol preparation, hereinafter called lactitol, was a preparation of lactitol monohydrate kindly supplied by CV Chemie Combinatie Amsterdam CCA (Gorinchem, The Netherlands), and the lactulose preparation, hereinafter called lactulose, was Duphalac, a syrup containing 0·67 g lactulose, 0·11 g galactose and 0·06 g lactose per ml (Duphar BV, Amsterdam, The Netherlands).

Determinations with pigs

Experimental animals

Six female miniature pigs of the Göttingen strain were used. They were 11 weeks old at the start of the experiment and weighed 3·4–7·0 kg.

Dietary treatments

In the first experiment the treatments were a control diet (a normal pig starter diet) and an experimental treatment in which lactitol was added to each meal of starter diet prior to feeding in the ratio 1 part lactitol to 10 parts starter diet. In both treatments, starter diet was provided on the same scale based on body weight (18 g food kg^{-1} body weight) and given as a slurry mixed with water. The daily ration was given in two halves, at 09.30 and 16.30 h. The composition of the starter diet (g kg^{-1}) was 249 barley meal, 163 fat mixture (Megalac, Volac Ltd, Royston, UK), 140 wheat meal, 134 soya bean meal, 110 skim milk powder, 110 white fish meal, 68 maize meal, 13 dicalcium phosphate, 5 sodium chloride, 4 trace mineral mixture, 4 vitamin mixture. The protein content (N × 6·25) was 218 g kg^{-1}. Water was available throughout the experiment.

In the second experiment the same starter diet was used and, again, given according to body weight. Supplements of a control solution (control treatment) or Duphalac (experimental treatment) were added to each meal before feeding. The amount of Duphalac depended on the tolerance of the individual pig and ranged from 276 to 465 ml kg^{-1} of starter diet. The control solution contained galactose and lactose in the same concentrations as in Duphalac, and its purpose was to equalise intakes of these sugars.

Housing

The pigs were individually housed at 22 C in specially designed metabolism cages which allowed separate and total collection of faeces and urine (Ratcliffe and Fordham 1987).

Experimental design

A cross-over design with two experimental periods was used. The pigs were divided into two equal groups (three pigs per group) using a table of random numbers. One group received experimental treatment and the other had the control treatment in the first experimental period; the treatments were switched for the second experimental period so that each pig was its own control. For lactitol (first experiment) the experimental periods lasted 7 days. The pigs were weighed on the first day of each period, and new levels of feeding were introduced according to changes in body weight. A 4-day period of adaptation to the diets then followed. The balances were started before the first feed on day 5 and lasted for 3 days—ending before the first feed on day 1 of the next experimental period. The treatments were then switched and the second adaptation period started. Separate collections of faeces and urine were made twice daily during the balance periods and stored in plastic bottles under 0·05 M sulphuric acid at 4°C. At the end of a balance, the bulked faeces and urine were stored at −20 C. In the second experiment (on lactulose) the adaptation period was extended to determine the maximum quantity of Duphalac each pig would consume with the production of soft faeces but without scouring. Otherwise, feeding and other experimental details followed the same routine as adopted in the first experiment.

Determinations with rats

Experimental animals

For the third experiment, 12 female rats were selected from the Laboratory's colony of Lister Norwegian hooded rats. The rats were 6 weeks old at the beginning of the experiment and weighed 150–170 g. Six female rats from the same colony were used in the fourth experiment. At the start they weighed 103–121 g.

Dietary treatments

In the third experiment the diets contained 900 parts by weight of a basal mixture:

maize starch 520 g
casein 119 g
ground sucrose 100 g
Solkafloc 50 g
maize oil 50 g
salt mixture (Achinewhu and Hewitt 1979) 50 g
vitamin mixture (US Pharmacopeia 1965) 10 g
Rovimix E_{250} (containing 250 mg α-tocopheryl acetate g^{-1}; Roche Products, Welwyn Garden City, Herts) 0·24 g
cyanocobalamin (in a solution containing 100 μg ml^{-1}) 2 μg

and 100 parts of maize starch in the control diet or 100 parts lactitol in the experimental diet. The same basal mixture was used in the fourth experiment. Each 900 parts of it was mixed with 50 or 75 parts of lactulose (in the form of Duphalac) and made up to 1000 parts with maize starch. The control diet contained 900 parts of basal, galactose and lactose, equivalent to the amounts in the Duphalac supplements, and maize starch to 1000 parts.

Throughout the third experiment the rats were given 12 g food daily at 09.30 h while in the fourth experiment they were given 10 g daily in the first period and 11 g in the second period.

Housing
During balance periods, the rats were individually housed in metabolism cages (Techniplast, Buguggiate, Italy) which allowed the separate and total collection of faeces and urine. Prior to this, the rats were individually housed in holding cages. The rats were kept in a constant temperature room at 22°C.

Experimental design
This was essentially the same as for the pig experiments. Sulphuric acid (2–3 ml 0·05 M) was placed in the vessel for collecting the urine to reduce losses of nitrogen. Contamination of the urine with food occurred very occasionally and was removed by filtering through Whatman filter paper (No 1).

Sample processing and analysis

The bulked faeces were freeze dried (Virtis Freeze-Drier model 50SR) and the dry weight was recorded. The dried faecal matter was then homogenised in a domestic food processor and stored in a desiccator over silica gel. Samples were taken for total N estimation by a micro-Kjeldahl method and for gross energy in an adiabatic bomb calorimeter (Gallenkamp, London) standardised using benzoic acid. The total urine collection was thawed, thoroughly mixed and weighed and a sample was removed for analysis of total N. Urine samples were prepared for bomb calorimetry by pipetting 10–20 ml on to a sheet of polythene film (cling film: 15 × 15 cm; Payne Scientific, Slough) placed in a 25-ml beaker and freeze drying. The dried urine sample was then wrapped in the polythene film and stored in a desiccator, also over silica gel. Pieces of polythene film were bombed separately so that corrections could be made for the energy released by their combustion. All analyses were carried out in duplicate. Gross energy values of diets, lactitol and freeze dried Duphalac were determined in triplicate or quadruplicate; N concentrations in the diets were also determined.

Calculations

Digestible energy value was calculated from the difference between gross energy values of the food consumed and the faeces collected, and it was expressed as kJ g^{-1} food. Metabolisable energy value was derived similarly, total excreta being considered rather than faeces alone. N-corrected metabolisable energy values were also calculated by correcting the energy balance by subtracting from it 28·33 kJ per g N retained in the case of the pig (Diggs *et al* 1965) and 26·33 kJ per g N retained in the case of the rat (Metta and Mitchell 1954).

From these values for the diets, energy values of lactitol and lactulose were calculated. For the pig, where test material was added to control starter diet, the following formula was used:

$$E = \frac{(1+i)E_c - E_c}{i}$$

where E is digestible (or metabolisable) energy value of test material (kJ g^{-1}); i is amount of test material added to 1 g of control diet (g); and E_e, E_c are digestible energy values of experimental and control diets, respectively (kJ g^{-1}).

In the rat experiment, the test materials replaced part of the maize starch in the control diet and so the following formula was appropriate:

$$E = E_s - \frac{E_c - E_e}{i}$$

where E_s is digestible (or metabolisable) energy value of maize starch (kJ g^{-1}); i is the concentration of test material in experimental diet (g g^{-1}); and E_e, E_c are as before. (Digestible energy value of maize starch is equal to its gross energy value of 17·48 kJ g^{-1} (Blaxter 1967); its metabolisable energy value is 16·58 kJ g^{-1}; Metta and Mitchell 1954.)

Statistical analysis

The data were subjected to standard analysis of variance for a cross-over experiment (Cochran and Cox 1957), and the standard errors of mean energy values for the diets are based on the residual error mean square with $r-2$ degrees of freedom where r is the number of animals. Each animal was its own control and generated an energy value for the test material. Mean values with standard errors for these energy values were also calculated.

RESULTS

In preliminary feeding trials with lactulose, rats showed a marked tendency to diarrhoea which made separation of faeces and urine impossible. For this reason, 75 g lactulose kg^{-1} diet was used for the experimental treatment as diarrhoea developed when the level of inclusion was 100 g kg^{-1}. A satisfactory adaptation to the experimental diet was achieved by feeding the rats a diet containing 50 g lactulose kg^{-1} for 2 days and then giving the experimental diet for 3 days before starting a balance. Lactulose was tolerated well by the pigs which consumed up to 310 g added to each kilogram of starter diet with few signs of diarrhoea. Lactitol was tolerated well by both pigs and rats, and an adaptation period was found unnecessary for the levels tested.

The results for individual animals in the nutritional balance studies are given in Tables 1–4 as the gross energy values of the food consumed and the excreta produced. The tables also give the digestible energy values and the metabolisable energy values, not corrected for nitrogen retention, derived from the intake and output data, and the corresponding values for lactitol and lactulose calculated from the energy values of the diets. Mean values with standard errors are given in Table 5. It should be noted that, by determination, the gross energy values of lactitol and lactulose were found to be 16·2 and 16·4 kJ g^{-1} respectively.

The experimental values for the diets were measured with high precision, as shown by coefficients of variation of 0·007–0·020, whereas for the derived values for the ingredients precision was of a lower order (coefficients of 0·12–0·38). This low

TABLE 1

Intake and excretion of gross energy (kJ day^{-1}) by growing miniature pigs fed on control diet and experimental diet containing lactitol, with digestible and metabolisable energy values (kJ g^{-1})

Pig number	*Diet*	*Energy intake*	*Faecal energy*	*Urinary energy*	*Digestible energy*		*Metabolisable energy*	
					Diet	*Lactitol*	*Diet*	*Lactitol*
19	Control	14001	2174	432	13·59	6·71	13·10	4·31
	Experimental	11582	2256	470	12·96		12·31	
21	Control	10525	1026	438	14·52	5·28	13·85	6·37
	Experimental	13495	2030	423	13·68		13·17	
23	Control	10525	1545	319	13·73	12·21	13·24	12·80
	Experimental	9563	1493	228	13·59		13·20	
25	Control	8690	1351	194	13·59	13·42	13·23	13·67
	Experimental	9563	1503	177	13·57		13·27	
27	Control	5214	788	115	13·66	9·02	13·31	9·24
	Experimental	5738	1020	106	13·24		12·94	
28	Control	6952	992	310	13·80	8·58	13·08	12·20
	Experimental	9563	1643	194	13·33		13·00	

TABLE 2

Intake and excretion of gross energy (kJ day^{-1}) by growing miniature pigs fed on control diet and experimental diet containing lactulose, with digestible and metabolisable energy values (kJ g^{-1})

Pig number	*Diet*	*Energy intake*	*Faecal energy*	*Urinary energy*	*Digestible energy*		*Metabolisable energy*	
					Diet	*Lactulose*	*Diet*	*Lactulose*
19	Control	9111	1125	222	14·10	8·53	13·71	6·87
	Experimental	8287	1213	310	13·26		12·68	
21	Control	7289	1115	255	13·62	12·34	13·06	11·57
	Experimental	10692	1494	403	13·29		12·70	
23	Control	7373	1125	325	13·63	7·90	12·92	8·29
	Experimental	8287	1402	294	12·90		12·35	
25	Control	5551	759	220	13·88	9·69	13·25	9·35
	Experimental	8620	1260	323	13·17		12·59	
27	Control	5635	980	254	13·29	6·67	12·56	6·92
	Experimental	6549	1355	236	12·20		11·65	
28	Control	5551	866	179	13·57	10·87	13·05	10·68
	Experimental	6882	1105	199	12·81		12·37	

TABLE 3

Intake and excretion of gross energy (kJ g $^{-1}$) by hooded rats fed on control diet and experimental diet containing lactitol, with digestible and metabolisable energy values (kJ g^{-1})

Rat number	*Diet*	*Energy intake*	*Faecal energy*	*Urinary energy*	*Digestible energy*		*Metabolisable energy*	
					Diet	*Lactitol*	*Diet*	*Lactitol*
1	Control	820·7	41·4	37·2	16·24	10·38	15·46	8·96
	Experimental	810·6	65·2	40·0	15·53		14·70	
2	Control	815·1	48·4	45·2	16·08	14·28	15·14	12·37
	Experimental	763·8	50·9	47·4	15·76		14·72	
3	Control	701·4	38·0	38·8	16·17	11·69	15·23	8·10
	Experimental	734·3	56·5	52·7	15·59		14·38	
4	Control	820·7	56·2	60·4	15·93	13·48	14·67	17·03
	Experimental	810·3	65·0	39·3	15·53		14·71	
5	Control	820·7	48·0	56·8	16·10	14·08	14·92	15·50
	Experimental	744·9	49·7	42·2	15·76		14·81	
6	Control	820·7	48·4	51·6	16·09	12·88	15·02	13·75
	Experimental	807·4	60·2	42·9	15·63		14·73	
7	Control	820·7	45·2	28·2	16·16	12·38	15·57	11·16
	Experimental	810·6	59·4	30·0	15·65		15·03	
8	Control	820·7	51·6	35·0	16·02	12·58	15·29	12·76
	Experimental	804·7	64·6	29·6	15·53		14·91	
9	Control	820·7	41·2	46·8	16·24	8·88	15·26	9·82
	Experimental	791·0	70·6	37·2	15·38		14·59	
10	Control	820·7	49·0	39·1	16·08	12·88	15·26	12·64
	Experimental	641·4	48·0	28·7	15·62		14·87	
11	Control	820·7	47·8	34·9	16·10	11·78	15·38	9·85
	Experimental	820·7	65·2	39·7	15·53		14·70	
12	Control	814·0	52·8	33·5	16·00	12·68	15·29	10·22
	Experimental	629·2	50·9	32·5	15·52		14·65	

TABLE 4

Intake and excretion of gross energy (kJ day $^{-1}$) by hooded rats fed on control diet and experimental diet containing lactulose, with digestible and metabolisable energy values (kJ g^{-1})

Rat number	*Diet*	*Energy intake*	*Faecal energy*	*Urinary energy*	*Digestible energy*		*Metabolisable energy*	
					Diet	*Lactulose*	*Diet*	*Lactulose*
1	Control	707·6	43·2	16·8	15·16	9·08	14·78	9·28
	Experimental	624·4	52·1	11·4	14·54		14·25	
2	Control	710·6	48·6	12·9	15·04	13·46	14·75	12·17
	Experimental	634·4	44·8	12·4	14·74		14·43	
3	Control	646·0	46·9	14·7	14·98	11·13	14·61	10·39
	Experimental	697·8	59·2	15·7	14·51		14·16	
4	Control	646·0	35·9	10·5	15·25	8·53	15·00	5·99
	Experimental	696·9	55·8	16·4	14·59		14·21	
5	Control	646·0	42·7	10·0	15·08	8·53	14·83	6·37
	Experimental	695·4	63·3	14·5	14·42		14·09	
6	Control	709·8	38·2	13·3	15·28	8·94	14·98	6·29
	Experimental	634·4	48·4	17·0	14·65		14·23	

TABLE 5

Energy values (kJ g $^{-1}$) of control and experimental diets determined with growing miniature pigs and hooded rats[a] and derived energy values of test materials, lactitol and lactulose

Test animal	*Test material*	*Energy value*	*Diet*			*Test material*	
			Control	*Experimental*	*SE*	*Mean*	*SE*
Miniature pig	Lactitol	Digestible	13·8	13·4	0·10	9·2	1·28
		Metabolisable	13·3	13·0	0·11	9·8	1·54
	Lactulose	Digestible	13·7	12·9	0·06	9·3	0·84
		Metabolisable	13·1	12·4	0·06	9·0	0·79
Hooded rat	Lactitol	Digestible	16·1	15·6	0·03	12·3	0·44
		Metabolisable	15·2	14·7	0·05	11·8	0·77
	Lactulose	Digestible	15·1	14·6	0·04	10·0	0·81
		Metabolisable	14·8	14·2	0·06	8·4	1·05

[a] Six animals per diet except that 12 rats were used for lactitol.

TABLE 6

Metabolisable energy values (kJ g^{-1}) of control and experimental diets corrected to zero N balance determined with growing miniature pigs and hooded rats[a] and derived energy values of test materials

Test animal	*Test material*	*Diet*			*Test material*	
		Control	*Experimental*	*SE*	*Mean*	*SE*
Miniature pig	Lactitol	12·9	12·6	0·11	10·0	1·52
	Lactulose	12·7	12·0	0·07	8·6	0·74
Hooded rat	Lactitol	15·1	14·6	0·04	11·6	0·61
	Lactulose	14·7	14·0	0·06	8·1	1·05

[a] Six animals per diet except that 12 rats were used for lactitol.

precision probably accounts for the unexpected result with pigs of a slightly higher value for the metabolisable energy of lactitol compared with its digestible energy value.

Table 6 shows metabolisable energy values corrected to zero N balance. Values for the diets were all slightly less than the uncorrected results (Table 5), more so in the pig than in the rat. The values derived for the test materials in Table 6 were little different from those obtained from the uncorrected energy values (Table 5), bearing in mind the lower precision.

DISCUSSION

The work described in this paper highlights one particular difficulty in the energy evaluation of poorly tolerated foodstuffs. The rats showed a tendency to diarrhoea when lactulose was tested, and to avoid this problem the levels of test materials were kept low. As a consequence the precision of the derived results was also low. This is because in the calculations (see pages 236 and 237) the level of inclusion is a divisor and, being small, it inflates the effect of any methodological or random errors. Further, there may be considerable animal-to-animal variation in the present experiments due to variation in the ability to digest and metabolise materials of the type tested. Any digestion probably depends on the activity of the gut flora within which there may be large differences between animals.

Although the amount of lactitol incorporated into the experimental diet was a rather small supplement for evaluation by the energy balance method, it is nevertheless representative of the amount that could be included in foods for man, and the results obtained here with rats and pigs may be applicable to the human. This remains to be fully investigated.

The reliability of the digestible and metabolisable energy values derived in this paper for lactitol and lactulose depends very much on the accuracy and precision of the gross energy values of the diet and excretory products which were determined by bomb calorimetry. As a check on this, the equation on page 237 for the rat results was used to calculate the gross energy values of lactitol and lactulose from the gross

energy values of the control and the respective experimental diets. This approach gave energy values (and SD) of 15·4 (1·4) kJ g^{-1} for lactitol and 11·1 (1·9) kJ ml^{-1} for Duphalac. These values compare with the determined values of 16·2 kJ g^{-1} and 12·5 kJ ml^{-1}, respectively. The calculated values are within 1 standard deviation of the determined ones, showing that the bomb calorimetry was reasonably accurate and precise. Such a check could not be applied to the determinations for pigs since their meals were prepared immediately before each feeding from appropriate quantities of control diet and test material.

The present results indicate that with lactitol considerable digestion of the test substance occurred and a similar picture emerged with lactulose. The ME values suggest that much of the digested energy was available for metabolic use by the pig or the rat. However, to ascertain the true extent of energy utilisation would require other experimental approaches. Product information supplied by the manufacturer of lactitol (CV Chemie Combinatie Amsterdam, CCA) gives a 'calorie utilisation' of lactitol in man of 8·4 kJ g^{-1} whereas in this work the metabolisable energy values of lactitol in rats and miniature pigs were found to be 11·8 and 9·8 kJ g^{-1} respectively. The energy value of lactitol was the subject of recent study in human volunteers (van Es *et al* 1986). In this report, an energy balance study indicated a metabolisable energy value of 80% of that of sucrose (saccharose). This is consistent with the data in this paper which indicate metabolisable energy values of 72 and 60% of gross energy values in the rat and miniature pig respectively.

Differences in energy values between man, rat and pig could be the result of differences in their gut microbial flora and its ability to break down lactitol. The porcine gut flora is in many respects similar to man's, but whereas the human stomach and upper small intestine are usually sterile, the stomach, duodenum and jejunum of pigs contain large numbers of bacteria. The most significant microbial activity in the porcine stomach is the fermentation of sugars, and this could represent an additional site for the digestion of lactitol in the pig. A difference between man and the rat that may be important is that the latter are known to practise coprophagy. Barnes *et al* (1957) estimated that even when rats are kept on wire mesh floors they recycle approximately 50% of their faeces. Despite considerable observation, particularly during the lengthy periods of time spent collecting the excreta, coprophagy was not seen during the experiments reported here, but it is nevertheless possible that a limited amount could have occurred.

Lactitol was originally proposed as a 'low-energy' sweetener and bulking agent with zero or nearly zero energy value. Investigations have shown that lactitol does contribute to the consumer's energy economy but there is insufficient data to be precise about the extent of this contribution. The ME values obtained in the present study suggest that lactitol may contribute too much energy to be of use in the manufacture of reduced-energy foods which in the UK must have no more than three-quarters of the energy value of the normal equivalent food. However, caution is required in drawing this conclusion since, as van Es *et al* (1986) proposed, the available energy may be considerably less than the ME value would suggest. Despite this uncertainty, lactitol does have certain other distinct characteristics which make it a useful ingredient for food technologists. The cariogenicity of lactitol is low (van der Hoeven 1986), so its use in products where it replaces sucrose may

contribute to a reduction in dental caries, particularly when it is used in the manufacture of chocolate, chewing gum, ice cream etc which are popular with children. Lactitol-containing foods may also be suitable for people suffering from diabetes, since the consumption of lactitol does not cause increased blood glucose or insulin levels (van Velthuijsen J A pers comm).

There do not appear to be any published energy values for lactulose in the literature. Its available energy has always been assumed to be small because it is not hydrolysed by homogenates of the human small intestinal mucosa (Dahlqvist and Gryboski 1965) and, on reaching the colon, it is metabolised by the bacteria to simple organic acids. The contribution these acids make to the energy balance of the host is a matter of continuing debate and investigation. However, the energy values reported here (8·4 and 9·0 kJ g^{-1} for the rat and pig respectively) suggest that the consumer may be able to derive more energy from lactulose than has been thought previously. This may be of interest to clinicians prescribing lactulose, particularly when it is used on a long-term basis to treat constipation in patients who may be bedridden or paraplegic and have problems limiting their energy intake.

ACKNOWLEDGEMENT

The support for one of us (SPB) from the Joint Committee of the Milk Marketing Board and the Dairy Trade Federation is gratefully acknowledged.

REFERENCES

Achinewhu S C, Hewitt D 1979 Assessment of the nutritional quality of proteins: the use of 'ileal' digestibilities of amino acids as measures of their availabilities. *Br J Nutr* **41** 559–571.

Barnes R H, Fiala G, McGehee B, Brown A 1957 Prevention of coprophagy in the rat. *J Nutr* **63** 489–498.

Bircher J, Müller J, Guggenheim P, Haemmerli U P 1966 Treatment of chronic portal-systemic encephalopathy with lactulose. *Lancet* **1** 890–893.

Blaxter K L 1967 *The Energy Metabolism of Ruminants*. Hutchinson, London.

Cochran W G, Cox G M 1957 *Experimental Designs* (2nd edn) John Wiley & Sons, New York.

Dahlqvist A, Gryboski J D 1965 Inability of the human small-intestinal lactase to hydrolyze lactulose. *Biochim Biophys Acta* **110** 635–636.

Diggs B G, Becker D E, Jensen A H, Norton H W 1965 Energy value of various feeds for the young pig. *J Anim Sci* **24** 555–558.

Mayerhofer F, Petuely F 1959 Untersuchungen zur Regulation der Darmatätigkeit des Erwachsenen mit Hilfe der Laktulose (Bifidus-Faktor). [Investigations regarding the regulation of intestinal activity in adults with the aid of lactulose (bifidus factor)]. *Wein Klin Wschr* **71** 865–869.

Metta V C, Mitchell H H 1954 Determination of the metabolizable energy of organic nutrients for the rat. *J Nutr* **52** 601–611.

Nilsson U, Jägerstad M 1987 Hydrolysis of lactitol, maltitol and Palatinit® by human intestinal biopsies. *Br J Nutr* **58** 199–206.

Ratcliffe B, Fordham J P 1987 A technique for rearing germ-free piglets obtained without surgery. *Lab Anim* **21** 53–59.

US Pharmacopeia 1965 *US Pharmacopeia* **XVII**. Mack Publishing Co, Easton, PA.

van der Hoeven J S 1986 Cariogenicity of lactitol in program-fed rats. *Caries Res* **20** 441–443.
van Es A J H, de Groot L, Vogt J E 1986 Energy balances of eight volunteers fed on diets supplemented with either lactitol or saccharose. *Br J Nutr* **56** 545–554.
van Velthuijsen J A 1979 Food additives derived from lactose: lactitol and lactitol palmitate. *J Agric Food Chem* **27** 680–686.

J Sci Food Agric 1990, **51**, 247–260

Prediction of Sensory Quality by Near Infrared Reflectance Analysis of Frozen and Freeze Dried Green Peas (*Pisum sativum*)

Lise Kjølstad,* Tomas Isaksson‡ and Hans J Rosenfeld

MATFORSK, Norwegian Food Research Institute, Osloveien 1, N-1430 Ås, Norway

(Received 14 December 1988; revised version received 10 July 1989; accepted 20 July 1989)

ABSTRACT

Frozen, green peas (Pisum sativum *L*) *of different varieties and different levels of maturity were evaluated by sensory analysis using a panel of ten trained judges. Two texture variables (hardness and mealiness) and four flavour variables (pea flavour, sweetness, fruity flavour, off flavour) were considered. Near infrared reflectance (NIR) analysis was performed with the same material, on both the frozen and freeze dried peas. The NIR instrument was calibrated to predict the sensory variables using the multivariate analytical method of principal component regression. Tenderometer readings of the same peas were also calibrated to predict the sensory variables. NIR analysis on the freeze dried peas showed relative ability of prediction (RAP) values for the sensory variables which were higher than those for the tenderometer readings. The sensory attributes pea flavour and hardness were predicted with higher RAP values by tenderometer readings than by NIR analysis on frozen peas.*

For the rest of the attributes, NIR analysis on frozen peas gave higher RAP values than tenderometer readings. NIR generally gave high RAP values, and this tentative study suggests that NIR analysis could be a useful tool in instrumentally assessing the quality of frozen peas.

Key words: Green peas, *Pisum sativum* L, frozen peas, freeze dried peas, near infrared reflectance (NIR), sensory analysis, principal component regression (PCR).

* Present address: Norwegian Food Control Authority, PO Box 8187 Dep, N-0034 Oslo 1, Norway.
‡ To whom all correspondence should be addressed.

J Sci Food Agric 0022-5142/90/$03.50

ABBREVIATIONS

NIR	Near infrared reflectance
PCA	Principal component analysis
PCR	Principal component regression
RMSP	Root mean square error of prediction
RMSCV	Root mean square error of cross-validation
RAP	Relative ability of prediction

INTRODUCTION

In Scandinavia frozen green peas are a common vegetable at the dinner table. In Norway the annual production of frozen peas has been about 5000 tonnes for the last 5 years. That means a production of about 1·2 kg per caput. The use of canned and dried peas is minor compared with that of frozen.

The sensory quality of peas may be assessed by laboratory studies using a panel of trained judges or by a consumer test (Amerine *et al* 1965). The relationship between product quality and the chemical/physical state of green peas has been studied by Blanchard and Maxwell (1941), Ottosson (1958), Atherton and Gaze (1983), Oleata *et al* (1983), Gaze *et al* (1986), Martens (1986) and others.

The yield and quality of green peas for processing primarily depends on their maturity at the time of harvest (Rutledge and Board 1980). Tenderometer readings are related to the maturity of the peas (Ottosson 1958; Rutledge and Board 1980; Rutledge 1981) and thereby are indirectly related to pea quality. Martens (1986) found that tenderometer value was a relevant but inadequate predictor of internal sensory quality. Atherton and Gaze (1983) recommended that product quality standards for frozen peas should not depend entirely on physical/chemical methods (tenderometry included), but that reference should also be made to sensory analysis of colour, flavour and texture.

Sensory analysis and traditional chemical analysis are often rather time consuming. A quick analysis with sufficient accuracy would save much time in assessing the quality of peas. Near infrared reflectance (NIR) has become a widely used method in food analysis because it is rapid and little or no sample preparation is required (Osborne and Fearn 1986). The technique has been used by many to predict the chemical constituents of peas. Williams *et al* (1978) applied NIR analysis to the determination of protein and moisture in a number of species of pulses and attained a squared coefficient of correlation of 0·89 to 0·96 between protein contents determined by Kjeldahl analysis and NIR analysis. Park *et al* (1982) found correlation coefficients between actual and NIR predicted values of 0·97 for crude protein, 0·77 for crude fat, 0·91 for ash and 0·81 for neutral detergent fibre in dehydrated vegetables. Davies and Wright (1984) determined the protein in pea flour with NIR analysis. The accuracy of the predictions was tested against different data sets and found to be better than 1·5 % ($P = 0·95$). Davies *et al* (1985) found that the accuracy of predictions of starch in pea flour was better than 2·6 % and of lipid 0·3 %. Williams *et al* (1985) used NIR to determine methionine and protein in whole

field peas and found the accuracy of prediction to be 0·11 and 0·76%, respectively. Tkachuk *et al* (1987) found that NIR analysis gave better prediction values for protein in ground than in whole field peas.

The sensory quality may be predicted by relating the sensory data to NIR data with the use of multivariate regression. Calibration of NIR reflectance data, by regression, to predict both sensory and chemical/physical properties has been done with frozen peas (Martens and Martens 1986). The measurements with an NIR instrument of 19 standard wavelengths (InfraAlyzer 400, Technicon Industrial Systems, Tarrytown, NY, USA) was then found to be better than tenderometer values at describing the average variation in sensory quality. The technique was also found to predict sensory texture variables better than in the case of the flavour variables. Martens and Martens (1986) concluded that NIR had the potential for predicting the sensory quality of frozen peas.

The 19 standard wavelengths of the InfraAlyzer 400 have been selected for the prediction of chemical constituents, but factors that contribute to the sensory perception of biological material are complex and multivariate. Therefore, by increasing the number of wavelengths, one may increase the information about sensory quality. In the present study the work of Martens and Martens (1986) has been continued with the use of a scanning NIR instrument. It was also of interest to find out if removal of the major part of the water in the peas would reduce the prediction error of the NIR analysis. Some possible reasons for improvement in predictive ability by removal of water are considered.

The flavour and texture quality of the peas was considered in this work since these aspects have been found to be important quality criteria for frozen peas. Schutz *et al* (1984) found that the internal sensory flavour and texture were more important for the purchase and consumption of vegetables than colour and appearanace. Martens (1986) found that the texture variables hardness and mealiness and the fruity and sweet flavour variables were relevant and representative internal sensory quality criteria for normally treated green peas. Sanford *et al* (1988) used principal component regression (PCR) to determine that the dimensions represented by texture and flavour were the most important factors in optimising consumer sensory acceptance of frozen peas. The development of sensory attributes for green peas has been described by Martens (1986). Only the internal sensory quality has been investigated in the present study and some alterations have been made: pea flavour was added to the analysis and the definition of fruity flavour was slightly changed.

EXPERIMENTAL

Materials

The materials used consisted of 60 independent batches of wrinkle-seeded, green peas (*Pisum sativum* L) from the 1987 season. The peas were collected from 27 varieties and at different degrees of maturity. From these batches samples of peas with a diameter of 9 mm or more were used in the study. The peas were blanched within 3 h of harvesting at 90–95°C for 2 min and cooled in tap water (~6°C). They were then frozen on perforated trays at −40°C (air velocity ~1·5 m s^{-1}) for at least

20 h (batches of 1–5 kg), packed in polyethylene bags and stored at −20°C (air velocity ~0·5 m s^{-1}).

About 70–90 g of whole peas from each batch of the frozen peas were freeze dried in a Hetosicc DC 13 freeze drier (Heto, Birkerød, Denmark).

Physical and chemical measurements

The maturity of the peas was assessed by measuring the hardness with an FMC tenderometer (Canners Machinery Ltd, Simcoe, Canada) immediately after harvesting.

Dry matter was determined by the freeze drying process. It was also determined by drying the frozen peas at 105°C for 16 h.

Sensory analysis

The sensory analysis was performed about 3 months after harvesting. The frozen peas were steam heated at 100°C before evaluation by a trained profile panel of 10 persons. Each of the 10 judges was served each sample twice. Portions of about 20 g were served in a randomised order within replicates and with respect to each judge.

Six sensory attributes were evaluated with the use of an intensity scale of 1 to 9 points. An intensity of 1 point represented no reference to the attribute, and 9 represented a very strong expression of it. The attributes were pea flavour (characteristic/rich), sweetness, fruity flavour (light/aromatic/fresh), off flavour (harsh/bitter/metallic), mealiness and hardness.

The analysis was performed with the use of the Senstec (Tecator, Höganäs, Sweden) registration system. Each judge evaluated a sample by marking an unstructured line of 15 cm for the intensity of each sensory attribute. Numerical values were obtained by automatic registration of the length from the left side of the line to the marking.

NIR analysis

The batches of both frozen and freeze dried peas were homogenised (Moulinette S643, Moulinex, Nieune, France) for about 60 and 30 s respectively prior to NIR analysis. The frozen peas were taken directly from the freezer for homogenisation.

The NIR analysis was performed with an InfraAlyzer 500 (Technicon Industrial Systems) at 2-nm intervals in the range 1100–2500 nm. The effective bandwidth was about 10 nm.

The homogenised samples were packed in standard black cups. An open cup (189-0822-01) was used for the frozen peas and a closed cup (189-B090-02) for the freeze dried material. The samples were measured at a temperature of about 22 C.

The detected diffuse reflectances (R) were transformed to apparent absorbances ($\log 1/R$). The mean spectrum of three repacks of each sample was used for calibration.

Data analysis

The number of datapoints in the spectra was decreased in order to reduce computing time by averaging every three adjacent datapoints starting with the first

three points and continuing with the next three etc. A selection of alternate averages, making a total of 116 variables, was entered into the data analysis.

The spectral data for each batch were then subjected to multiplicative scatter correction (Martens *et al* 1983) to reduce nonlinear scatter effects. Previous works with wheat (Martens *et al* 1983) and meat (Geladi *et al* 1985; Isaksson and Næs 1988) have demonstrated that simpler calibration models can be obtained with the use of multiplicative scatter correction.

The multivariate data analytical method PCR (Gunst and Mason 1979; Cowe and McNicol 1985) was used for calibration. With this method the variables are transformed to eigenvectors called principal components using a principal component analysis (PCA) algorithm (Wold *et al* 1984). The regressions are performed with these principal components as independent variables and the sensory attributes as dependent variables. The principal components were incorporated into a regression model in decreasing order of their eigenvalue (Næs and Martens 1988). Spectral data from frozen and freeze dried peas and tenderometer readings from fresh peas were subjected to calibration.

To validate the calibration model the PCR was performed with the use of cross-validation (Stone 1974). The cross-validation was performed with four segments (calibration repeated four times), each time treating a quarter of the entire calibration sample set as a test set (prediction samples) while the rest of the samples were used for calibration. As a result all the calibration samples were treated as prediction samples once.

The predictive accuracy of a calibration model is described by the root mean square error of prediction (RMSP) (Martens and Martens 1986) which is defined for the sensory variable y_j by:

$$\mathrm{RMSP} = \sqrt{\frac{1}{I}\sum_{i=1}^{I}(\hat{y}_i - y_i)^2}$$

where y_i is the sensory data from analysis of sample number i, $\hat{y}_i$ is the predicted value, and I is the number of objects used in the prediction.

An average of RMSP^2 for all cross-validation segments was computed for each principal component model. The square root of this average is called the root mean square error of cross-validation (RMSCV) (Martens and Naes 1987).

In order to compare the predictive ability of the calibration models for the different sensory variables, the relative ability of prediction (RAP) was used (Martens and Martens 1986). This term takes into account the level of experimental error in the reference data. RAP is defined for sensory variable y_j by:

$$\mathrm{RAP} = \frac{S_{\mathrm{tot}}{}^2 - \mathrm{RMSCV}^2}{S_{\mathrm{tot}}{}^2 - S_{\mathrm{ref}}{}^2}$$

where S_{tot} is the standard deviation of the reference data y_j in all the samples. S_{ref} is a standard error that indicates the uncertainty of the analysis due to the judges, and is defined for sensory variable y_j by:

$$S_{\mathrm{ref}} = \sqrt{\frac{1}{IN}\sum_{i=1}^{I} S_i^2}$$

where S_i is the standard deviation of the points (each point is the mean of two replicates) from the judges at sample number i, I is the number of samples, and N is the number of replicates for each judge. By removal of S_{ref}, the RAP equation will express the squared multivariate correlation coefficient (Martens and Næs 1987).

The predictive ability explained by RAP will have a value between 0 and 1. A RAP value of 0 means that the prediction error, RMSCV, is equal to S_{tot}, while 1 means that the prediction is perfect and the RMSCV is equal to S_{ref}. An increase in S_{ref} increases the RAP, but the S_{ref} will never exceed the error of prediction expressed as RMSCV.

The transformations and regressions were executed with the software Unscrambler version 2.0 (CAMO A/S, Trondheim).

RESULTS AND DISCUSSION

Results from the sensory analysis, tenderometer measurements and analysis of dry matter are given in Table 1. The mean value ($\bar{y}$), range, standard deviation (S_{tot}) and standard error due to experimental noise (S_{ref}) are shown. The peas were chosen to span a wide range of variation with respect to maturation which also gave a large quality variation. S_{tot} and S_{ref} were used in the calculations of RAP.

The correlation coefficients between the different sensory attributes are given in Table 2. There is a high intercorrelation between the variables.

Martens (1986) found quality criteria covering the variation caused by maturation and growing season that seemed to be valid independent of variety. Ottosson (1958) found only small differences between varieties measured with chemical methods. Variation due to different varieties was therefore not considered in this study.

TABLE 1

Results from the sensory analysis, tenderometer readings and analysis of dry matter. Mean of 60 pea batches ($\bar{y}$), range total standard deviation (S_{tot}) and the experimental noise standard error (S_{ref})

	$\bar{y}$	*Range*	S_{tot}	S_{ref}
Pea flavour	5·4	2·3–7·3	1·1	0·29
Sweetness	5·5	2·2–7·2	1·2	0·25
Fruity flavour	3·6	1·2–5·2	1·0	0·31
Off flavour	2·9	1·8–6·6	1·0	0·29
Mealiness	4·4	2·2–6·8	1·3	0·33
Hardness	4·8	2·6–8·0	1·4	0·27
Tenderometer	135·2	88–200	31	—
Dry matter[a] (g kg^{-1})	223	179–288	25	—
Dry matter[b] (g kg^{-1})	195	138–281	31	—

[a] Dried at 105°C for 16 h.
[b] Freeze dried.

TABLE 2
Correlation coefficients between the different sensory attributes

	Sweetness	*Fruity flavour*	*Off flavour*	*Mealiness*	*Hardness*
Pea flavour	0·94	0·97	−0·94	−0·93	−0·92
Sweetness	—	0·94	−0·89	−0·90	−0·94
Fruity flavour	—	—	−0·90	−0·96	−0·93
Off flavour	—	—	—	0·83	0·85
Mealiness	—	—	—	—	0·92

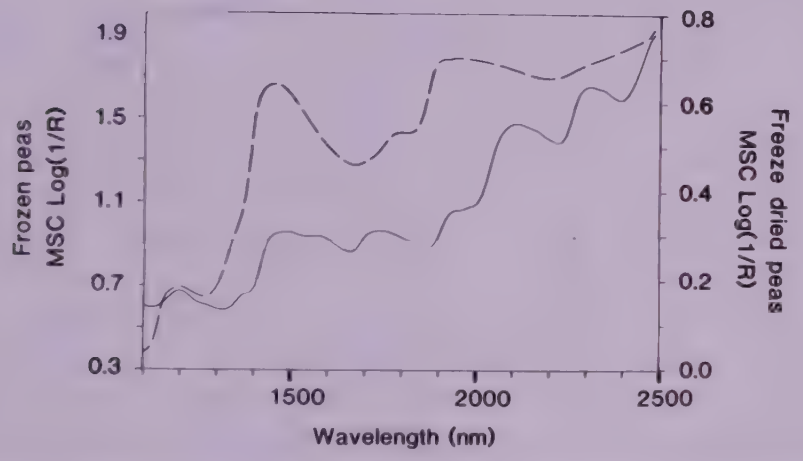


Fig 1. An example of NIR spectra of sample with a tenderometer value of 110. Spectra of both the freeze dried (——) and frozen (– –) samples are illustrated. The absorbance scale (log $1/R$) for frozen peas is to the left of the diagram while that to the right is for freeze dried peas.

Examples of NIR spectra for a pea sample are shown in Fig 1. Spectra from both the frozen and freeze dried samples are illustrated. The freeze dried peas had lower log ($1/R$) values and more distinct peak widths.

By removal of water, the level of absorbance will decrease. This is partly due to less absorption of energy by water. The peaks become more distinct when there is less overlapping by water absorbance. Also, there is more difference between the refractive indices of the particles and air than between the particles and water. The reflection will increase when the difference in the refractive indices increases.

Homogenisation produced smaller particles of the freeze dried material than homogenisation of the frozen, which gave more reflection surfaces. Increased reflection leads to smaller 'diffuse thickness' (Birth 1978) of the material. The path of radiation before the light is reflected back to the detector becomes shorter, and consequently there will be less absorption of radiant energy.

Both spectra had peaks at absorption bands for water (1450 nm and 1940 nm). Other peaks of interest in the spectra may be the 1520–1570 and 2100–2120 nm areas, in which there are absorption bands for carbohydrates (Osborne and Fearn 1986). The amount of carbohydrates in peas is quite high. Martens (1986) found levels of starch of about 5·0% and of sugar of about 7·5% in 96 different pea samples from three different seasons.

The RAP made it possible to compare the ability of NIR to predict the different sensory variables. To be able to see the development with different calibration models, the RAP values are presented in Fig 2 as functions of the number of principal components. RAP values with NIR analysis (frozen and freeze dried samples) and tenderometry as predictors are illustrated.

The maximum RAP value with NIR analysis of freeze dried peas was higher than with tenderometer readings. Compared with the predictive ability of NIR on frozen peas, the tenderometer gave a higher maximum RAP value for pea flavour and slightly higher for hardness. NIR analysis on frozen peas showed higher RAP values for the remaining attributes. A tenderometer reflects variations due to resistance to compression and extrusion (Rutledge 1981), but on the whole it did not reflect the variation in internal sensory quality as well as the NIR.

Tenderometer readings had the lowest prediction errors when predicting hardness with a RAP value of 0·91 and fruity flavour with 0·86. The tenderometer value was correlated to hardness ($r = 0{\cdot}94$) and fruity flavour ($r = -0{\cdot}89$). A relatively high ability of prediction expressed as RAP was therefore not unexpected. With an increasing tenderometer value, the hardness will increase and the fruity flavour will weaken mainly because of the decreasing amount of sugar, increasing amount of starch and less water.

The maximum predictive ability, expressed as RAP, for freeze dried peas was higher than for frozen peas except when predicting off flavour. The RAP values of off flavour were about equal in the two different NIR analyses. The high RAP values for the freeze dried peas demonstrate that information about the sensory quality was improved by the removal of water. The absorbance level decreased and the chemical constituents contributed relatively more to the spectra. There is a possibility of structural damage during freeze drying if a critical temperature is exceeded (Bellows and King 1972). Possibly structures and/or chemical bondings are changed in such a way that the specific absorbances explained more about the attributes when built into a calibration model. Some of the components or structures that either contribute to off flavour or are correlated to it may have been destroyed during the freeze drying process.

Sweetness, fruity flavour, hardness and mealiness were the best predicted sensory attributes for both NIR analyses. The freeze dried material showed maximum RAP values between 0·93 and 0·98, while the values of the frozen material ranged from 0·88 to 0·91. Pea flavour was also among the best in NIR analysis of the freeze dried material (0·93). Off flavour had the lowest RAP values with both the freeze dried and frozen material with maximum values of 0·84 and 0·83, respectively. This accuracy in predicting sensory attributes suggests that NIR could be a useful tool in assessing the quality of peas. Even NIR analysis of frozen peas gave information about the quality with relatively high accuracy.

Martens and Martens (1986) related sensory data for frozen peas to NIR data (19 standard wavelengths) by partial least squares regression. They found a predictive ability, expressed as RAP, for texture variables (0·79) higher than for flavour variables (0·67). In the present work there was no tendency for prediction ability of the flavour variables (pea flavour, fruity flavour, sweetness, off flavour) to be lower than that of the texture variables (hardness, mealiness).

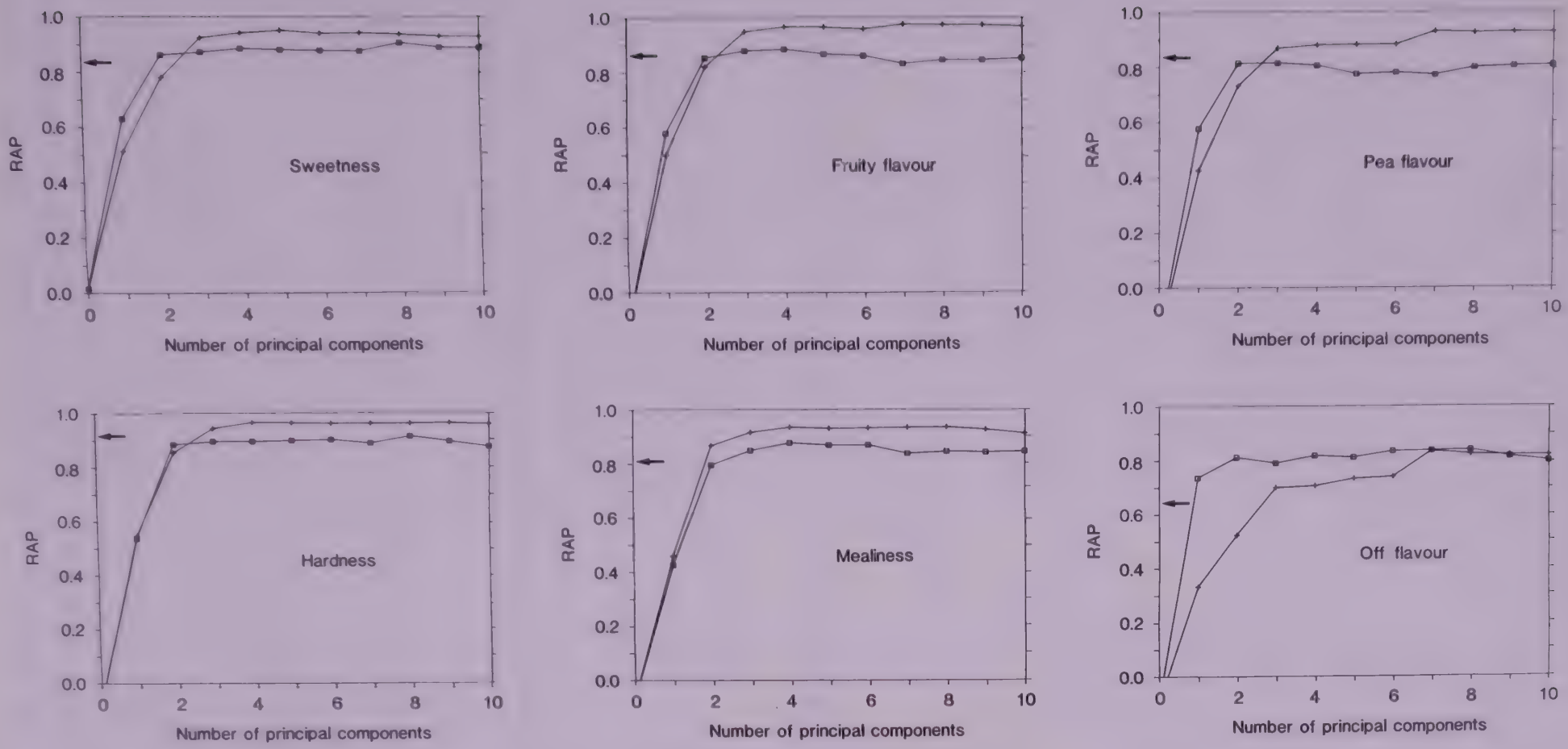


Fig 2. The relative ability of prediction (RAP; see text) illustrated as a function of the number of principal components in the regression models. Six different internal sensory attributes are predicted by both NIR analysis and tenderometer readings. RAP for NIR analysis of both freeze dried and frozen peas are illustrated (+ freeze dried peas, □ frozen peas). RAP values for tenderometer reading are indicated with arrows.

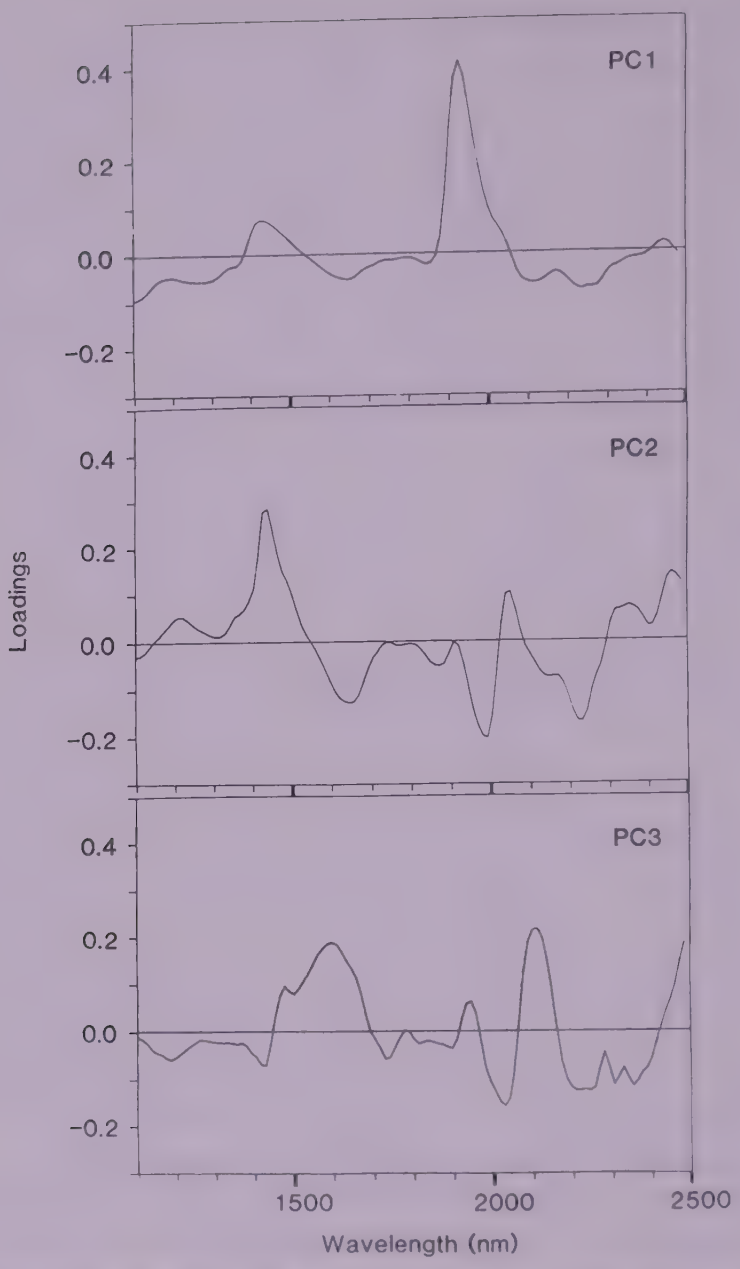


Fig 3. Principal component loadings at each datapoint of the NIR spectrum of freeze dried peas. The principal components PC1, PC2 and PC3 are numbered according to decreasing eigenvalues.

An increased number of wavelengths possibly increased the ability to predict flavour variables compared with the texture variables. This improvement has to be verified by a comparative study of the two NIR instruments using the same regression technique and the same material for analysis.

Loading plots of the three first principal components of freeze dried peas and the two first principal components of frozen peas are illustrated in Figs 3 and 4. For freeze dried peas, the three first principal components contributed to the major predictive ability with all the sensory attributes. The first principal component generally contributed more than the second, which contributed more than the third.

The first and second principal components after analysis of freeze dried peas showed positive correlation to hardness and negative correlation to sweetness. The loading plots of the first and second principal components are similar to the water

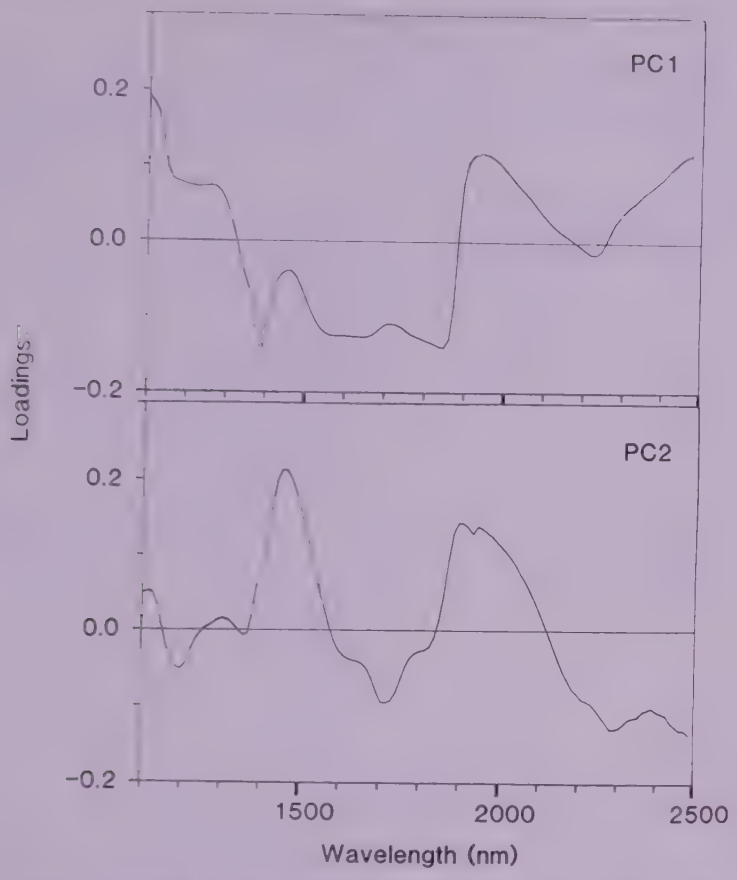


Fig 4. Principal component loadings at each datapoint of the NIR spectrum of frozen peas. The principal components PC1 and PC2 are numbered according to decreasing eigenvalues.

and carbohydrate spectra (Williams and Norris 1987), respectively. The third principal component was positively correlated to sweetness and negatively correlated to hardness, and the loading plot had a structure which resembled that of a carbohydrate spectrum, possibly amorphous sucrose (Davies and Miller 1988).

For frozen peas, only the two first principal components contributed to the major predictive ability. Both of the loading plots resemble the NIR water spectrum and have only slight structures of carbohydrate spectra. The first and second principal components were then likely to span most of the water variation.

The spectra from freeze dried peas contained relatively more information about sensory attributes than did the spectra from frozen peas. The information seems to have been collected from more fine structured principal components related to carbohydrates.

The content of starch increases and that of sugar decreases during maturation of the peas (Ottosson 1958) and indicates that the amount of carbohydrates is important for the quality. A tentative interpretation would also be that the water binding ability, here represented with the remaining water after freeze drying, is important for the sensory quality.

The minimum values of the prediction errors expressed as RMSCV, with NIR and tenderometer readings as predictors, are given in Table 3. The freeze dried peas gave lower prediction errors than frozen peas for all sensory variables except for off flavour which had an RMSCV value only slightly higher than that of the frozen

TABLE 3

Minimum of root mean square error of cross-validation (RMSCV; see text) with NIR analysis and tenderometer readings as predictors for sensory quality attributes

	NIR		*Tenderometer readings*
	Freeze dried	*Frozen*	*Fresh*
Pea flavour	0·40 (7)	0·54 (3)	0·52
Sweetness	0·36 (5)	0·44 (8)	0·54
Fruity flavour	0·34 (7)	0·45 (4)	0·47
Off flavour	0·49 (7)	0·48 (8)	0·64
Mealiness	0·46 (4)	0·55 (4)	0·64
Hardness	0·37 (4)	0·49 (8)	0·48

( ) Number of principal components which gave the lowest prediction errors.

peas. The tenderometer readings gave higher prediction errors expressed as RMSCV than those of the freeze dried peas.

Lower RMSCV values for freeze dried as opposed to frozen peas mean greater accuracy and suggest that more information about the material was retrieved when the major part of the water was removed. The prediction then comes closer to the 'true' value measured by sensory analysis.

Further studies are needed to make calibration models for use by producers and the processing industry. Pea samples must be chosen to represent the normal range of variation with respect to varieties and maturities. Also seasonal effects need to be included by calibration with peas from more than one year. Instruments suitable for field and industrial use have to be selected, or constructed if not available.

CONCLUSIONS

NIR analysis gave higher predictive ability than tenderometer readings for most of the internal sensory texture and flavour variables of frozen peas. NIR analysis of freeze dried peas gave better predictive ability of texture and flavour for frozen peas than NIR analysis on frozen peas.

ACKNOWLEDGEMENTS

Part of this work was supported by the Agricultural University of Norway under valuable guidance from Professor Kjell Steinsholt. Marit Rødbotten, Sigrid Hurv and Laura Blümlein are thanked for the execution of the sensory analysis. We would also like to thank Bjørg Narum Nilsen and Liv Bakke for technical assistance and typing, respectively.

REFERENCES

Amerine M A, Pangborn R M, Roessler E B 1965 *Principles of Sensory Evaluation of Food*. Academic Press, New York.

Atherton G, Gaze R R 1983 Sensory quality of frozen peas in relation to maturity measurements. In: *Refrigeration in the Service of Man, XVIth Int Congr Refrigeration*, Paris, Vol 3'G. Institut International du Froid, Paris, pp 581–587.

Bellows R J, King C J 1972 Freeze-drying of aqueous solutions: maximum allowable operating temperature. *Cryobiology* **9** 559–561.

Birth G S 1978 The light scattering properties of foods. *J Food Sci* **43** 916–925.

Blanchard E L, Maxwell M L 1941 Correlations of subjective scoring with sugar content of frozen peas. *Food Res* **6** 105–115.

Cowe I A, McNicol J W 1985 The use of principal components in the analysis of near-infrared spectra. *Appl Spectrosc* **39** 257–266.

Davies A M C, Miller C E 1988 Tentative assignment of the 1440-nm absorption band in the near-infrared spectrum of crystalline sucrose. *Appl Spectrosc* **42** 703–704.

Davies A M C, Wright D J 1984 Determination of protein in pea flour by near infrared analysis. *J Sci Food Agric* **35** 1034–1039.

Davies A M C, Coxon D T, Gavrel G M, Wright D J 1985 Determination of starch and lipid in pea flour by near infrared reflectance analysis. The effect of pea genotype on starch and lipid content. *J Sci Food Agric* **36** 49–54.

Gaze R R, Atherton D, Holdsworth S D 1986 Suitability of the Ottawa pea tenderometer to assess the quality of raw peas. *Food Technol* **21** 319–329.

Geladi P, MacDougall D, Martens H 1985 Linearization and scatter-correction for near-infrared reflectance spectra of meat. *Appl Spectrosc* **39** 491–500.

Gunst R F, Mason R L 1979 Some considerations in the evaluation of alternate prediction equations. *Technometrics* **21** 55–63.

Isaksson T, Næs T 1988 The effect of multiplicative scatter correction (MSC) and linearity improvement in NIR spectroscopy. *Appl Spectrosc* **42** 1273–1284.

Martens H, Næs T 1987 Multivariate calibration by data compression. In: *Near-infrared Technology in the Agricultural and Food Industries*, ed Williams P C & Norris K. American Association of Cereal Chemists, St Paul, Minn, pp 57–87.

Martens H, Jensen S Å, Geladi P 1983 Multivariate linearity transformations for near-infrared reflectance spectrometry. In: *Nordic Symposium on Applied Statistics*, ed Christie O H J. Stokkand Forlag Publ., Stavanger, pp 205–233.

Martens M 1986 Sensory and chemical/physical quality criteria of frozen peas studied by multivariate data analysis. *J Food Sci* **51** 599–603 and 617.

Martens M, Martens H 1986 Near-infrared reflectance determination of sensory quality of peas. *Appl Spectrosc* **40** 303–310.

Næs T, Martens H 1988 Principal component regression in NIR analysis: viewpoints, background details and selection of components. *J Chemometrics* **2** 155–167.

Oleata J A, Montgomery M W, Cain R F 1983 Effect of maturity on the physical, chemical and sensorial properties of frozen peas. *Agric Tec (Santiago)* **43** 53–60.

Osborne B G, Fearn T 1986 *Near Infrared Spectroscopy in Food Analysis*. Longman Scientific & Technical, London.

Ottosson L 1958 *Growth and Maturity of Peas for Canning and Freezing*. Almqvist & Wiksells Boktryckeri AB, Uppsala.

Park Y W, Anderson M J, Mahoney A W 1982 Potential of near infrared reflectance (NIR) spectroscopy for estimating nutrient content of dehydrated vegetables. *J Food Sci* **47** 1558–1561.

Rutledge P J 1981 Effect of harvesting procedures on measurement of maturity in peas. *CSIRO Food Res Q* **41** 75–79.

Rutledge P J, Board P W 1980 Comparison of physical methods for estimating the maturity of raw, frozen and cooked peas. *Texture Stud* **11** 379–388.

Sanford K A, Gullett E A, Roth V J 1988 Optimization of the sensory properties of frozen peas by principal components analysis and multiple regression. *Can Inst Food Sci Technol J* **21** 174–181.

Schutz H G, Martens M, Wilsher B, Rødbotten M 1984 Consumer perception of vegetable quality. *Acta Hort (The Hague)* **163** 31–38.

Stone M 1974 Cross-validatory choice and assessment of statistical prediction. *J R Stat Soc B* **36** 111–133.

Tkachuk R, Kuzina F D, Reichert R D 1987 Analysis of protein in ground and whole field peas by near-infrared reflectance. *Cereal Chem* **64** 418–422.

Williams P C, Norris K (ed) 1987 *Near-infrared Technology in the Agricultural and Food Industries*. American Association of Cereal Chemists, St Paul, Minn, pp 247–288.

Williams P C, Stevenson S G, Starkey P M, Hawtin C 1978 The application of near infrared reflectance spectroscopy to protein-testing in pulse breeding programmes. *J Sci Food Agric* **29** 285–292.

Williams P C, Mackenzie S L, Starkey P M 1985 Determination of methionine in peas by near-infrared reflectance spectroscopy (NIRS). *J Agric Food Chem* **33** 811–815.

Wold S, Albano C, Dunn III W J, Edlund U, Esbensen K, Geladi P, Hellberg S, Johansson E, Lindberg W, Sjøstrøm M 1984 Multivariate data analysis in chemistry. In: *Chemometrics. Mathematics and Statistics in Chemistry*, ed Kowalski B R. Reidel Publishing Company, Dordrecht, pp 17–95.

J Sci Food Agric 1990, **51**, 261–269

Influence of Age on Muscle Connective Tissue in Trout (*Salmo irideus*)

Pilar Montero and Javier Borderías*

Instituto del Frío, Ciudad Universitaria, 28040 Madrid, Spain

(Received 27 April 1989; revised version received 7 July 1989; accepted 31 July 1989)

ABSTRACT

The physicochemical properties of the connective tissue in the muscle of trout (Salmo irideus *Gibb*) *from four different age groups were studied. Solubility, thermal denaturation and the yields of the α,β and γ components were used as indices of collagen aggregation. Shear strength of connective tissue from different locations in trout fillets was also measured. Muscle connective tissue in the larger trout (1800 g) was more abundant and the collagen exhibited a lower level of crosslinking. However, shear strength values were significantly different only for the youngest specimens (200 g). Collagen crosslinking increased with size in the other three groups studied (200, 800 and 1200 g); the connective tissue from the 800-g and 1200-g specimens had similar shear strength values which were higher than those for the 200-g specimens.*

Key words: Fish, muscle, collagen, connective tissue, age, trout.

INTRODUCTION

The influence of age on fish collagen has received very little attention and consequently most publications refer to higher vertebrates. The research carried out to date attests to differences of opinion about the alterations taking place in animal collagen with age. Some workers have reported collagen contents increasing with age (Hunsley *et al* 1971; Bailey *et al* 1974; Nakamura *et al* 1975; Bornsteins and Traub 1979; Wada *et al* 1980) whereas others have found only a slight relationship in muscle connective tissue (Reagan *et al* 1976) or dermal connective tissue (Hall *et*

* To whom correspondence should be addressed.

J Sci Food Agric 0022-5142/90/$03.50

al 1974). On the other hand, Kurosu (1979) reported that the amount of muscle collagen decreased as animals grew older.

Kruggel and Field (1974) observed an increase in the amount of acid-soluble collagen with age but also pointed out that there were more chains, that is fewer crosslinks, in the collagen of older animals than in that of young animals. They concurred with Schaub's theory (1963) that in older animals collagen formation takes place in the striated muscle and that this newly formed collagen has fewer crosslinks. Love *et al* (1982) measured the amount of cod (*Gadus morhua*) collagen soluble in citric acid and concluded that the degree of crosslinking was higher in older individuals than in young fish but that the difference was very slight. They attributed this to synthesis during the starvation period in cod of new collagen which is subsequently reabsorbed when food is plentiful with consequent regular replenishment of the collagen.

A more recent study by Bogason (1984) on rockfish (*Sebastes* spp) indicated that the collagen from the individuals analysed exhibited a high level of crosslinking and that this may have been due to their longevity. However, Bogason acknowledged that further study was required (Bogason 1984).

The objective of the present work was to compare the biochemical and physical characteristics of muscle connective tissue in trout (*Salmo irideus*) in four different age groups. In addition to the scientific interest of studies of this nature, the results are important in their bearing on both processing technology and the development of commercial applications for connective tissue.

MATERIALS AND METHODS

Trout (*Salmo irideus* Gibb) reared on a fish farm were used, so providing lots consisting of individuals of similar size. The total weights of the lots were 7·2, 4·8, 3·2 and 1·2 kg, the mean length of individual fish was 46·0, 42·5, 40·0 and 23·3 cm, and the mean weight was 1800, 1200, 800 and 200 g, respectively. The age range in each of these lots was 22–25, 20–22, 19–21 and 10–12 months, respectively. Specimens were refrigerated in ice from the time of capture until use at the laboratory some 24 h later.

Separation and purification of connective tissue

The fish were headed and gutted, and the connective tissue was separated and purified using the method of Borderías and Montero (1985).

Collagen solubility

A modified version of the method of fractionation in saline and acid solutions of Timpl *et al* (1975) was employed, as described by Borderías and Montero (1985). All determinations were performed in duplicate.

Quantitative collagen analysis

Hydroxyproline, an amino acid found almost exclusively in collagen, was used as the indicator for the quantitative determinations. Samples were hydrolysed with an

equal volume of 6 M hydrochloric acid at 110°C for 24 h in a sealed test tube. The hydrolysates were then dried in a vacuum desiccator and dissolved in distilled water. The hydroxyproline content of the resulting solutions was measured according to the method of Leach (1960). Measurements were in triplicate.

Determination of the α, β and γ components

Molecular sieve chromatography was performed in duplicate employing the techniques described by Chandrakasan *et al* (1976) and Krieg *et al* (1981) as modified by Borderías and Montero (1985).

Thermal denaturation

The method of thermal denaturation applied was based on the method developed by Sekoguchi *et al* (1979). An 8 g litre^{-1} NaCl solution was added to the purified connective tissue in the proportion of 10 ml of solution to 1 g of sample. The mixture was allowed to stand at room temperature for 1 h. It was then heated in a water bath at 35°C for 1 h; test tubes were sealed to prevent evaporation. Samples were then centrifuged at 4000 × *g* for 30 min. The hydroxyproline present in the supernate and in the precipitate was determined as indicated in the preceding section. Results have been expressed as the percentage of soluble collagen in relation to total collagen. Four replications of all determinations were performed.

Shear strength of connective tissue

Fish fillets devoid of remnants of skin, fat and bones were sliced lengthwise into 2·5-cm-wide strips of similar size. In the 200-g specimens, one lengthwise strip was cut from the dorsal half and another from the ventral half of each fillet; in the remaining specimens fillets yielded two dorsal and two ventral strips each, an inner strip and an outer strip.

Shear strength was measured transversely to the longitudinal axis of the strip in an Instron Model 1140 texturometer using a Warner Bratzler shear cell (Bratzler 1949). The last peak before the yield point was taken as the connective tissue peak. The first peaks corresponded to the muscle fibres, while the last peak was very sharp and much more pronounced, corresponding solely to the connective tissue (Moller 1980–1981). The results, the means of four replications, have been expressed in Newtons (N).

Statistical analyses

The standard deviation was calculated for the yield of connective tissue from the muscle, collagen solubility (salt-soluble and insoluble fractions), the proportions of the α, β and γ components in the acid-soluble fraction, and the soluble collagen values after thermal denaturation. Two-way analysis of variance using Tukey's test was also applied to establish the degree of significance for differences between the means.

The standard deviation of the shear strength values for the connective tissue from the different size specimens was also calculated. The significance of differences between the means for the shear strength of the connective tissue from fillets from individuals in different size groups was determined by a *t*-test.

RESULTS AND DISCUSSION

Table 1 presents the yields of connective tissue from trout muscle from the various age groups. Differences between the three youngest size groups were very slight, and the proportion of connective tissue rose substantially only in the case of the largest trout (1800 g). This increase in muscle collagen in long-lived individuals is explicable in terms of the theory advanced by Schaub (1963) who reported muscle collagen synthesis in very old animals such that collagen levels could be similar to, or even higher than, those in very young animals. Love *et al* (1982) recorded negligible differences in cod between the amounts of muscle collagen in the oldest and youngest specimens and pointed out that this may have been because collagen was synthesised during the starvation periods, thereby periodically replenishing collagen levels.

Collagen solubility in the different size groups is shown in Table 2. The acid-soluble (in 0·5 M acetic acid) collagen was the most abundant fraction in all the groups. For the salt-soluble fraction the differences were significant between all the

TABLE 1
Yields of connective tissue from the muscle of trout in different size groups (g kg^{-1})

Sample group	*Age (months)*	*Connective tissue in the muscle*
200 g	10–12	18 ± 3^{a}
800 g	19–21	14 ± 3^{a}
1200 g	20–22	$13{\cdot}5 \pm 1^{a}$
1800 g	22–25	33 ± 3^{b}

[a,b] Different letters indicate significant differences ($P \geq 0{\cdot}05$).
Results are the means of five determinations.

TABLE 2
Muscle collagen solubility in trout in different size groups (% total collagen)

Sample group	*SS*	*AS*	*I*
200 g	$2{\cdot}6 \pm 0{\cdot}2^{a}$	$70{\cdot}4 \pm 2{\cdot}4^{a}$	$27{\cdot}1 \pm 1{\cdot}2^{a}$
800 g	$5{\cdot}2 \pm 0{\cdot}0^{b}$	$66{\cdot}1 \pm 0{\cdot}5^{b}$	$28{\cdot}6 \pm 0{\cdot}6^{b}$
1200 g	$5{\cdot}9 \pm 0{\cdot}3^{c}$	$66{\cdot}1 \pm 1{\cdot}3^{b}$	$28{\cdot}0 \pm 1{\cdot}0^{b}$
1800 g	$1{\cdot}4 \pm 0{\cdot}1^{d}$	$74{\cdot}1 \pm 0{\cdot}7^{c}$	$24{\cdot}2 \pm 0{\cdot}6^{c}$

SS = Salt-soluble collagen fraction; AS = acid-soluble collagen fraction; I = insoluble collagen fraction.
[a–d] Different letters in each column indicate significant differences ($P \geq 0{\cdot}05$).
Results are the means of two determinations.

TABLE 3
Proportion of α, β and γ components in the acid-soluble collagen from trout in different size groups (% total components)

Sample group	*Component*		
	α	β	γ
200 g	$48{\cdot}7 \pm 0{\cdot}1^a$	$36{\cdot}0 \pm 0{\cdot}2^a$	$15{\cdot}4 \pm 0{\cdot}5^a$
800 g	$40{\cdot}8 \pm 1{\cdot}4^b$	$35{\cdot}9 \pm 1{\cdot}6^a$	$24{\cdot}1 \pm 1{\cdot}0^b$
1200 g	$36{\cdot}9 \pm 0{\cdot}3^c$	$28{\cdot}6 \pm 0{\cdot}9^c$	$44{\cdot}2 \pm 2{\cdot}3^c$
1800 g	$39{\cdot}8 \pm 2{\cdot}0^b$	$49{\cdot}2 \pm 0{\cdot}7^d$	$10{\cdot}9 \pm 0{\cdot}0^d$

$^{a-d}$ Different letters in each column indicate significant differences ($P \geqslant 0{\cdot}05$).
Results are the means of two determinations.

TABLE 4
Proportion of soluble collagen in trout after thermal denaturation (% total collagen)

Sample group	*Soluble collagen*
200 g	$49{\cdot}81 \pm 0{\cdot}9^a$
800 g	$19{\cdot}31 \pm 0{\cdot}5^b$
1200 g	$15{\cdot}92 \pm 0{\cdot}7^b$
1800 g	$45{\cdot}79 \pm 1{\cdot}3^c$

Different letters indicate significant differences ($P \geqslant 0{\cdot}05$).

lots, but for the acid-soluble and insoluble fractions the differences between the 800- and 1200-g lots were not significant ($P \geqslant 0{\cdot}05$). A number of investigators have reported that muscle collagen solubility levels decreased with age (Gantayat and Patnaik 1980; Bogason 1984; Feinstein and Buck 1984). This decrease was not very pronounced in the trout, and in the oldest specimens solubility values rose once more. Referring again to Schaub's (1963) theory, this may have been caused by the presence of recently synthesised collagen with fewer crosslinks.

Table 3 gives the proportions of the α, β and γ components in the acid-soluble collagen fraction. The proportion of monomers and dimers was generally higher than that of components with higher levels of crosslinking, providing support for the high percentage of soluble collagen shown in Table 2. The proportion of γ components increased with age, except in the oldest trout in which it dropped. This may have been because recently synthesised collagen has fewer crosslinks, as already pointed out above (Schaub 1963; Love *et al* 1982).

Table 4 sets out the data on the thermal denaturation of collagen with age. There were significant differences between the solubility values for the different age groups after thermal denaturation of the collagen. The collagen became more insoluble with age, except in the oldest specimens, in which solubility levels climbed again.

TABLE 5

Connective tissue shear strength values at different locations between the head and the tail ir fillets from trout in different size groups (Newtons)

	200-g trout	
	Dorsal strip	*Ventral strip*
HEAD END		
	1·70 ± 0·1	2·02 ± 0·0
	1·88 ± 0·3	2·03 ± 0·1
	1·65 ± 0·2	1·87 ± 0·0
	1·98 ± 0·3	1·80 ± 0·0
	1·90 ± 0·1	1·83 ± 0·2
	1·95 ± 0·1	2·02 ± 0·2
	2·04 ± 0·5	2·13 ± 0·7
	3·48 ± 0·1	3·10 ± 0·2
	5·13 ± 0·3	3·21 ± 0·1
	5·06 ± 0·2	3·21 ± 0·4
	5·23 ± 0·1	4·10 ± 0·3
TAIL END		

	800g trout			
	Dorsal strips		*Ventral strips*	
HEAD END				
	6·0 ± 0·5	28·0 ± 1·4	9·0 ± 1·0	3·5 ± 0·3
	6·5 ± 0·1	30·0 ± 0·7	11·0 ± 0·7	4·5 ± 0·5
	9·0 ± 0·2	31·0 ± 0·5	12·0 ± 0·4	4·5 ± 0·0
	10·5 ± 0·2	38·0 ± 0·5	14·0 ± 0·9	4·0 ± 0·0
	6·0 ± 0·2	28·0 ± 0·7	26·5 ± 0·5	2·5 ± 0·0
	4·0 ± 0·0	27·0 ± 1·0	21·0 ± 1·1	5·0 ± 0·7
	5·5 ± 0·1	28·5 ± 0·9	22·0 ± 0·8	5·0 ± 0·5
	5·5 ± 0·1	28·0 ± 0·5	24·0 ± 0·3	6·0 ± 0·4
	9·0 ± 0·1	34·0 ± 1·0	22·0 ± 1·3	6·5 ± 0·9
	7·5 ± 0·1	47·0 ± 1·4	31·0 ± 4·0	6·0 ± 0·1
	16·0 ± 0·5	51·0 ± 3·4	23·0 ± 1·3	4·5 ± 0·2
	14·5 ± 0·3	61·0 ± 2·3	28·0 ± 1·2	6·5 ± 0·2
	24·5 ± 2·2	60·0 ± 2·9	20·0 ± 4·1	6·0 ± 0·6
TAIL END				

Each figure is the mean of four determinations.

This was consistent with the results for the solubility of the salt-soluble and acid-soluble collagen fractions and for the proportions of the α, β and γ components (Tables 2 and 3). In contrast, in the oldest trout the collagen was more soluble and had fewer crosslinks. As mentioned previously, this was probably because it had recently been synthesised.

The correlation coefficient for the collagen insoluble in the acid solution and the collagen insolubilised by heat treatment was 0·716 at the 99% level of significance, indicating a relationship between these two parameters.

Table 5 illustrates shear strength as measure of the hardness of the connective

TABLE 5—*contd*

	1200-g trout			
	Dorsal strips		*Ventral strips*	
HEAD END				
	11·0±1·4	13·5±1·2	15·0±1·0	11·5±1·3
	10·0±0·7	13·0±0·1	13·5±0·7	10·5±0·5
	9·0±0·4	16·5±0·2	14·4±0·5	11·0±1·0
	7·0±0·6	10·5±1·0	14·5±1·1	9·0±0·7
	5·5±0·0	11·0±1·9	13·0±1·1	8·0±0·3
	8·0±0·1	12·5±0·7	13·0±0·9	8·5±0·2
	6·5±0·1	16·0±1·3	12·5±0·7	7·5±0·3
	5·5±0·5	11·0±0·5	18·5±0·9	9·0±1·0
	5·0±0·3	31·0±0·5	11·5±1·1	10·0±0·9
	5·5±0·2	30·0±0·3	21·5±0·3	17·5±3·4
	7·5±0·4	36·0±2·0	35·0±1·3	19·5±3·3
	7·0±0·4	23·0±0·5	40·0±2·0	16·5±0·9
	7·5±0·3	12·5±0·9	45·0±1·0	16·0±0·3
	14·5±1·7	18·0±0·4	45·0±0·5	15·0±1·0
	13·0±0·0	28·5±1·3	47·0±1·9	15·0±0·6
	17·0±0·7	16·0±1·6	54·0±2·8	16·5±0·8
TAIL END				
	1800-g trout			
	Dorsal strips		*Ventral strips*	
HEAD END				
	21·5±1·5	17·0±1·0	18·5±0·7	4·0±0·1
	20·5±0·9	11·0±2·0	14·5±0·9	4·0±0·1
	19·5±1·3	16·5±0·6	16·0±0·3	6·0±0·0
	16·0±0·5	16·5±0·3	17·0±0·9	6·0±0·1
	12·0±0·8	15·0±0·7	18·5±0·8	7·5±0·3
	20·0±2·0	20·5±1·1	17·5±0·3	7·5±0·2
	15·0±0·6	13·0±0·1	17·5±0·1	9·0±1·1
	6·0±0·4	25·5±2·3	19·5±0·3	9·0±0·7
	7·0±0·7	37·0±3·0	20·0±0·7	14·0±0·9
	6·0±0·1	30·0±1·9	18·0±0·5	16·0±1·5
	6·5±0·4	31·0±2·1	23·5±2·0	17·0±1·5
	3·5±0·4	27·5±0·9	25·0±1·7	17·0±0·9
	4·0±0·3	21·0±1·3	27·5±2·0	18·5±2·5
	9·5±2·0	23·0±2·0	26·5±1·0	17·5±1·9
	10·5±1·2	18·5±1·1	37·5±1·8	20·0±1·7
	9·5±0·1	23·5±0·7	54·5±3·4	21·5±1·0
	12·5±1·2	28·5±1·1	54·0±1·0	20·0±1·8
TAIL END				

Each figure is the mean of four determinations.

tissue at different points along the trout fillets in the different size groups tested. The youngest specimens exhibited the lowest hardness values for the muscle connective tissue. The hardness of the connective tissue increased towards the tail, though not as distinctly in the oldest trout. Hardness values tended to be somewhat higher in

TABLE 6

Significance (*t*-test) of differences between shear strength values for the connective tissue in trout of different size groups

Sample:	*800 g*				*1200 g*				*1800 g*			
Sample:	*D1*	*D2*	*V1*	*V2*	*D1*	*D2*	*V1*	*V2*	*D1*	*D2*	*V1*	*V2*
200 g	*	*	*	*	*	*	*	*	*	*	*	*
800 g	—	—	—	—	—	*	3*	*	—	2*	—	*
1200 g					—	—	—	—	—	2*	—	—

* $P \geq 0{\cdot}01$; 2* $P \geq 0{\cdot}05$; 3* $P \geq 0{\cdot}1$.

D1, Outer dorsal strip; D2, inner dorsal strip; V1, inner ventral strip; V2, outer ventral strip.

the dorsal half of the trout fillets than in the ventral half. Table 6 shows the degree of significance between mean shear strength values in different parts of the trout fillets in the various size groups. There were significant differences at the 99% level between the youngest specimens and the other three age groups. Differences between the specimens in the 800-g, 1200-g and 1800-g size groups were smaller (Table 6).

The reason the connective tissue in the oldest trout did not seem to become softer, even though larger amounts of connective tissue were present and the level of crosslinking was lower, was probably because, despite the presence of very soft, newly synthesised collagen with few crosslinks, there remained enough highly aggregated collagen to give high shear strength values.

REFERENCES

Bailey A J, Robins S P, Balian G 1974 Biological significance of the intramolecular cross-links of collagen. *Nature* **251** 105–106.

Bogason S G 1984 Characterization of the intramuscular connective tissue collagen in the three rockfish species (*Sebastes*). PhD Thesis, Oregon State University. University Microfilms International, Ann Arbor, Michigan.

Borderías A J, Montero P 1985 *Changes in Fish Muscle Collagen during Frozen Storage. Storage Lives of Chilled and Frozen Fish and Fish Products*. International Institute of Refrigeration, Aberdeen, pp 85–91.

Bornsteins P, Traub W 1979 The chemistry and biology of collagen. In: *The Proteins* (3rd edn), Vol 4, eds Neurath H & Hill R H. Academic Press, New York.

Bratzler L J 1949 Measuring the tenderness of meat by the use of the Warner Bratzler method. In: *Proc 2nd Reciprocal Meat Conf 2*. American Science Association, Chicago, IL, pp 117–121.

Chandrakasan G, Torchia D A, Piez K A 1976 Preparation of intact monomeric collagen from rat tail tendon and skin and the structure of the non-helical ends in solution. *J Biol Chem* **251** (19) 6062–6067.

Feinstein G R, Buck E M 1984 Relationship of texture to pH and collagen content of yellowtail flounder and cusk. *J Food Sci* **49** 298–299.

Gantayat S C, Patnaik B K 1980 Collagen characteristics of skin and muscle of the ageing teleost, *Ophiocephalus punctatus*. *Exp Gerontol* **15** 375–377.

Hall D A, Reed F B, Nuki G, Vince J D 1974 Relative effect of age and corticosteroid therapy

on the collagen profiles of dermis from subjects with rheumatoid arthritis. *Age Ageing* **3** 95–99.

Hunsley R E, Vetter R I, Kline E A, Burroughs W 1971 Effect of age and sex on quality, tenderness, and collagen content of bovine *longissimus* muscle. *A Anim Sci* **33** 933–935.

Krieg T, Luderschmidt C, Weber L, Muller P K, Braun-Falco O 1981 Sclerodermal fibroblasts: some aspects of *in vitro* assessment of collagen synthesis. *Dermatol Res* **270** 263–277.

Kruggel W G, Field R A 1974 Collagen characteristics of pronghorn antelope and sheep muscle. *J Food Sci* **39** 639–641.

Kurosu H 1979 Biochemical studies on collagen and connectin from human skeletal muscle. Age-related changes in the properties of elasticity. *Nippon Seikeigeka Gakkai Zasshi* **53** (11) 1641. *Chem Abstr* **92** 1081102.

Leach A A 1960 Notes on a modification of the Newman and Logan method for the determination of the hydroxyproline. *Biochem J* **74** 62–70.

Love R M, Lavety J, Vellas F 1982 Unusual properties of the connective tissues of cod (*Gadus morhua* L.). In *Chemistry and Biochemistry of Marine Food Products*. AVI Westport, CT, pp 67–72.

Moller A J 1980–1981 Analysis of Warner–Bratzler shear pattern with regard to myofibrillar and connective tissue components of tenderness. *Meat Sci* **5** 247–260.

Nakamura R, Segoguki S, Sato Y 1975 The contribution of intramuscular collagen to the tenderness of meat from chicken with different ages. *Poult Sci* **54** 1604–1606.

Reagan J O, Carpenter Z L, Smith G E 1976 Age-related traits affecting the tenderness of the bovine *longissimus* muscle. *J Anim Sci* **43** 1198–1200.

Schaub M C 1963 The aging of collagen in the striated muscle. *Gerontol* **8** 16–20.

Sekoguchi S, Nakamura R, Sato Y 1979 Cysteamine-induced changes in the properties of intramuscular collagen and its relation to the tenderness of meat obtained from mature chickens. *Poultry Sci* **58** (5) 1213–1216.

Timpl R, Ganville R, Nowack H, Wiedemann H, Fietzek P, Kühn K. 1975 Isolation, chemical, and electron microscopical characterization of neutral-salt-soluble type III collagen and procollagen from fetal bovine skin. *Hoppeseiler's Z-Physiol-Chem* **356** 1783–1792.

Wada K, Shirai K, Kawamura A 1980 Properties of collagens from pigskins of different ages. *J Amer Leather Chem Assoc* **75** 90–95.

J Sci Food Agric 1990, **51**, 271–274

Quantitative Determination of Suberin Deposition during Wound Healing in Potatoes (*Solanum tuberosum* L)*

Narakkat P Sukumaran, Jagdish S Jassal and Sirish C Verma

Central Potato Research Institute, Simla 171001, India

(Received 20 July 1989; accepted 12 September 1989)

Key words: Suberin, wound healing, potato, apical region, basal region, middle region.

Quantitative determination of suberin deposition during wound healing of potatoes (Solanum tuberosum *L*) *has shown that at RH* > *90 % a maximum amount of suberin was deposited at 25°C. A significantly greater amount of suberin was deposited at the basal region of the tuber as compared with the apical and middle regions.*

Proper healing of wounds of potato tubers prevents water loss through evaporation and prevents entry of rot-causing microorganisms. A number of investigations have therefore been done to study wound healing (see, for earlier references, Wigginton 1974). It comprises wound periderm formation and the deposition of a polymeric substance, suberin. Earlier investigations have relied upon histological examination to monitor the progress of wound healing (Artschwager 1927; Wigginton 1974; Thomas 1982). The extent of suberisation as judged in a section of the tissue (Wigginton 1974; Thomas 1982) is at best subjective. Thus there was felt to be a need for a procedure to provide a quantitative estimate of the amount of suberin deposited during wound healing in potatoes. We report here such a procedure based on the method described by Kolattukudy and Dean (1974) for the preparation of polymeric material from the wound periderm of potato.

Potato (*Solanum tuberosum* L) varieties (see Table 1) were grown either at the Central Potato Research Station (CPRS), Kufri (77°10′E 31°06′N, altitude 2430 masl) during April to September, or at CPRS, Modipuram (77°43′E, 29°01′N, altitude 225 masl), Meerut District during October–November to February–March. The crop at CPRS Kufri was rainfed and at CPRS, Modipuram it was irrigated.

Ten tubers of each variety were surface sterilised. Tubers were cut longitudinally (transversely at appropriate places in experiments to determine suberin deposition in different regions of the tubers) with a sterilised stainless steel knife and kept in

* Publication No 1165, Central Potato Research Institute, Simla, India.

TABLE 1
Suberin deposition at different temperatures in potato varieties

Varieties	*Suberin (mg cm^{-2})*				*Mean*
	Temperature (°C)				
	20	*25*	*30*	*35*	
Kufri Chandramukhi	4·6	8·6	4·0	2·8	5·0
Kufri Jyoti	5·0	6·8	3·1	3·3	4·5
Kufri Lalima	5·0	5·1	3·1	3·4	4·2
Kufri Sindhuri	5·5	7·9	3·3	3·4	5·4
Mean	5·0	5·6	3·4	3·2	
			LSD ($P \leqslant 0{\cdot}01$)		
Temperatures			0·82		
Varieties			0·68		
Between two variety means at the same temperature					2·15
Between two temperature means of the same or a different variety					1·41

humidity cabinets maintained at appropriate temperatures. After 7 days the suberised layer was removed and two discs of a known area (3–4 cm^2) were taken from this layer.

The isolation of suberin from the discs was done essentially according to the procedure described by Kolattukudy and Dean (1974) for the preparation of polymeric material from the wound periderm.

Twenty discs were treated with a solution of commercial preparations of cellulase and pectinase in 0·05 M acetate buffer, pH 4·0, for 40 h. The discs were then transferred to a 2:1 mixture of chloroform and methanol, and kept for a similar period. The discs were dried and weighed.

The data were subjected to statistical evaluation. For the experiments to determine the effect of temperatures on suberin deposition, a split plot design was used. The temperatures were assigned to main plots and the varieties were in sub-plots. For the experiments to determine the extent of suberin deposition in different regions, the data for two varieties used were pooled and analysis of variance was done using the randomised block design.

Our preliminary experiments confirmed earlier observations (Artschwager 1927; Wigginton 1974; Thomas 1982) that the process of suberisation is essentially complete in 7 days at 90% relative humidity. All experiments reported here were therefore done at a minimum relative humidity of 90%. Results of an experiment carried out with tubers of four varieties grown at CPRS, Modipuram, are summarised in Table 1. Maximum suberin deposition occurred at 25 °C. Both varieties and temperatures differed significantly at the 1% level. In a similar experiment done at 15, 20 and 25 °C with tubers of three varieties grown at CPRS, Kufri, maximum suberin deposition was also observed at 25 °C. In this test also, there was a significant effect of both variety and temperature (data not presented).

To study differences in the amount of suberin deposited in different parts of the tuber, 10 tubers each of varieties Kufri Chandramukhi and Kufri Lalima were cut

transversely at the apical, basal and middle regions of the tuber. The amount of suberin deposited in 7 days at 20°C and RH 90% was determined. It was observed that the amount of suberin deposited at the basal region (5·35 mg cm^{-2}) was significantly more (LSD P 0·01 = 0·45) than that at the middle (4·40 mg cm^{-2}) and apical (4·45 mg cm^{-2}) regions.

Our results are in general agreement with findings published earlier (Priestley and Woffenden 1923; Wigginton 1974; Thomas 1982) in that the maximum suberin deposition occurred at 25°C. The results do not, however, agree with the observations of Karmarkar and Joshi (1941) who found a greater degree of suberisation at higher temperatures up to 32°C. Thomas (1982) did not find any difference in suberisation between 25°C and 28–30°C, but at 35°C it was lower than at 25°C, as observed by us also.

A number of workers have observed varietal differences in wound healing in air in potatoes (Priestley and Woffenden 1923; Artschwager 1927; Smith and Smart 1955). Such differences in the quantity of suberin deposited have been observed by us in respect of some of the Indian potato varieties.

We did not observe any differences in the amount of suberin deposited at the apical region and at the middle of the tuber. To this extent our results confirm those of Reeve *et al* (1963). However, a significantly higher level of suberin deposition at the heel end does not agree with the result of Reeve *et al* (1963). These differences could be due to the different varieties used by us.

From the results presented here it is concluded that the amount of suberin deposited during wound healing in potatoes can be determined quantitatively by a simple procedure described by Kolattukudy and Dean (1974) and that the procedure reveals the differences between varieties as due to the temperature at which wound healing occurred.

ACKNOWLEDGEMENT

We are grateful to Mr S M Verma and Mr Jagpal Singh, Scientists-in-Charge, Central Potato Research Station, Modipuram, for the supply of tubers.

REFERENCES

Artschwager E 1927 Wound periderm formation in the potato as affected by temperature and humidity. *J Agric Res* **35** 995–1000.

Karmarkar D V, Joshi B M 1941 Investigations on the cold storage of potatoes. Indian Council of Agricultural Research Bull No 45. ICAR, New Delhi.

Kolattukudy P E, Dean R B 1974 Structure, gas chromatographic measurement and function of suberin synthesised by potato tuber tissue slices. *Plant Physiol* **54** 116–121.

Priestley J H, Woffenden L M 1923 The healing of wounds in potato tubers and their propagation by cut sets. *Ann Appl Biol* **10** 96–115.

Reeve R M, Forrester L J, Hendel C E 1963 Histological analyses of wound healing in potatoes treated to inhibit sprouting. II. Puncture wounds and *Fusarium* inoculates. *J Food Sci* **28** 655–762.

Smith W L Jr, Smart H F 1955 Relation of soft rot development to protective barriers in Irish potato slices. *Phytopath* **45** 649–654.

Thomas P 1982 Wound induced suberisation and periderm development in potato tubers as affected by temperature and gamma-irradiation. *Potato Res* **25** 155–164.

Wigginton M J 1974 Effects of temperature, oxygen tension and relative humidity on the wound healing process in the potato tuber. *Potato Res* **17** 200–214.

J Sci Food Agric 1990, **51**, 275–279

Fluoride Content of Some Common South Indian Foods and Their Contribution to Fluorosis

K Venkateswara Rao and Chaman L Mahajan

Department of Biosciences, Sri Sathya Sai Institute of Higher Learning, Prasanthinilayam 515134, India

Key words: Fluorosis, food, water, rural population, South India, fluoride content.

Fluoride contents of 98 food items commonly used in South India were determined with special reference to the feeding habits of remote rural populations in 41 villages of Anantapur District, Andhra Pradesh, India, where dental and skeletal fluorosis is endemic. Fluoride up to 4·5 mg kg^{-1} was found in irrigation and drinking water supplies. Thirty-two locally grown food items had generally higher fluoride contents (ranging from 0·2 to 11·0 mg kg^{-1}) with the notable exception of coconut water where even traces of fluoride could not be detected. The combined daily intake of fluoride from food and drinking water in the local population was found to range from 2·2 mg to 7·3 mg (0·05–0·32 mg kg^{-1} BW). The role played by food composition, cooking habits of the local population, general poverty and illiteracy in contributing to the prevalence of fluorosis in schoolchildren in the age group 6–18 years (even in areas with drinking water supplies within the permissible levels as per WHO standards) is discussed.

The area investigated is between 30° 40′–15° 15′ N and 76° 50′–78° 30′ E with an average rainfall of 544 (±98) mm, and temperature ranging from 15·7°C to 42°C. Mean maximum temperature is 36·4°C. It is considered one of the driest parts of South India. Because of the arid and drought-prone nature of the area, the population is generally poverty stricken with rice as the staple food.

A preliminary survey of the incidence of fluorosis in schoolchildren revealed the presence of dental fluorosis even in areas where the fluoride content of available drinking water supplies was within the generally accepted permissible level of 1·4 mg litre^{-1} and below (WHO 1984). This fact prompted the present study of the fluoride content in common food items of daily use to determine the possible contribution by foods, particularly those locally grown when considered together with the fluoride content of the water supply. Ninety-eight food items analysed have been represented as 15 sets of histograms arranged according to the fluoride content (Fig 1). The food samples were collected from the local market and analysed using an Orion fluoride meter (model 409; Orion Research, Cambridge, Mass, USA). Published procedures were used for sample preparation: leafy vegetables (Villa

J Sci Food Agric 0022-5142/90/$03.50

Fig 1. Fluoride content of common foods. Calculations based on average of three samples; limits of variation for all items within $\pm 10\%$ of the mean; food items determined on fresh weight basis; 7, 11, 12, 14, 17, 18, 35, 36, 66–98; food items determined on dry weight basis: 1–6, 8–10, 13, 15, 16, 19–34, 37–65. The limits of determination of the method used were 0·01–10 mg kg^{-1}. Analytical quality controls applied were: (i) ashing procedure was avoided to prevent any loss of fluoride during processing; (ii) polyethylene ware was used for handling extracts to avoid any possible leaching from glassware; (iii) reagents used (including distilled water) for processing in extraction and dilution procedure were fluoride free; (iv) a rigorous control of the reagent blank was maintained throughout the estimation.

Key to Fig 1

Tea
1 Lamsa
2 A1 Dust
3 Three Roses
4 Red Label
5 Taj Mahal
6 Super Dust

Miscellaneous foods
7 Baking powder
8 Sago
9 Betel leaf
10 Bread
11 Milk chocolate
12 Plain chocolate
13 Poppy seeds
14 Jaggery
15 Table salt
16 Cane sugar
17 Sugar cane juice
18 Coconut water

Leafy vegetables
19 Paruppu keerai
20 Curry leaf
21 Coriander leaf
22 Fenugreek leaf
23 Tamarind leaf
24 Ambat chuka
25 Amaranth, tender
26 Drumstick leaves

Spices and condiments
27 Cardamom
28 Cloves
29 Omum
30 Turmeric
31 Cumin seeds
32 Cinnamon
33 Pepper
34 Coriander

Milk products
35 Raptokos
36 Amul

Cereal grain products
37 Rice
38 Jowar
39 Broken wheat
40 Wheat flour
41 Wheat, refined
42 Ragi
43 Bajra
44 Maize

Pulses and legumes
45 Field bean
46 Peas
47 Bengal gram dhal
48 Soya bean
49 Cowpea
50 Red gram, dhal
51 Green gram, dhal
52 Black gram, dhal

Coffee
53 Nescafe
54 Bru Cafe
55 Gouri Sankar
56 Bru
57 Green Label
58 Beans, powdered

Nuts and oil seeds
59 Mustard seed
60 Almond
61 Cashew nut
62 Ground nut

Other vegetables
63 Broad bean
64 Drumstick
65 Cluster bean

Fruit
66 Banana
67 Orange
68 Lemon, sweet
69 Papaya, ripe
70 Melon, musk
71 Melon, water
72 Apple
73 Lemon
74 Grape
75 Tomato, ripe

Other vegetables
76 Brinjal
77 Pumpkin
78 Cabbage
79 Bottle gourd
80 Onion stalks
81 Cauliflower
82 Chochomarrow
83 Cucumber
84 Mango, raw

Roots and tubers
85 Sweet potato
86 Carrot
87 Beetroot
88 Onion
89 Radish
90 Potato

Spices and condiments
91 Tamarind
92 Garlic
93 Chillies
94 Ginger

Milk
95 Goat
96 Dairy (a mixture of buffalo's and cow's milk supplied by Government Dairy after pasteurisation)
97 Buffalo
98 Cow (fresh milk tested immediately after milking; unpasteurised)

TABLE 1
Estimated daily intake of fluoride from food and drinking water[a]

Age (years)	*Body weight (kg)*	*Daily fluoride intake (mg)*			
		Drinking water[b]	*Food*[c]	*Total*	*Per kg BW*
Pre-school children					
1–3	8–16	0·9–1·3	1·3	2·2–2·6	0·14–0·32
4–6	13–24	1·2–1·7	1·3	2·5–3·0	0·11–0·24
School children					
7–9	16–35	1·5–2·1	1·6	3·1–3·7	0·09–0·23
10–12	25–54	1·9–2·7	1·9	3·8–4·6	0·07–0·18
Boys					
13–18	40–67	2·2–3·2	2·5	4·7–5·7	0·07–0·14
Girls					
13–18	36–57	1·7–2·6	2·1	3·8–4·7	0·07–0·13
Adult (male)					
Sedentary		1·8–2·7	2·3	4·1–5·0	0·05–0·09
Moderate activity	54–85	2·1–3·0	2·7	4·8–5·7	0·06–0·11
Heavy work		2·7–3·9	3·4	6·1–7·3	0·07–0·14
Adult (female)					
Sedentary		1·6–2·4	1·8	3·4–4·2	0·05–0·09
Moderate activity	45–74	1·7–2·6	2·1	3·8–4·7	0·05–0·10
Heavy work		2·2–3·2	2·7	4·9–5·9	0·07–0·13

[a] After McClure (1949).
[b] Calculated on the basis of an average of 2·3 mg litre^{-1} F for the area investigated (Venkateswara Rao and Mahajan 1989).
[c] Calculated on the basis of data on food consumption from Gopalan *et al* 1982.

1970), tea (Cook 1970), milk (Beddows and Kirk 1981), other food materials (Dabeka *et al* 1979). Fluoride in fruit juices and coconut milk was determined directly. The fluoride contents of the foods are presented as histograms in Fig 1. Locally grown food items in areas where the fluoride content of water used for irrigation was above 1·5 mg litre^{-1} were rice, field beans, onion, groundnut, broad bean, drumstick, cluster bean, brinjal, bottle gourd, onion stalks, tamarind pulp, chillies, ginger, papaya, water melon, ripe tomato, betel leaf, milk, jaggery, sugar cane juice, coconut water and all leafy vegetables. Other food items tested were generally grown in other parts of the country outside the study area but constitute daily items of food in the area.

The estimated total daily intake of fluoride by different age groups is given in Table 1. It is evident from the estimated fluoride intake (1·3 to 3·4 mg) that the food contributes significantly to the endemic fluorosis mentioned above. A similar contribution through food has been reported for fluorotic areas elsewhere such as Texas, USA (WHO 1970). This would explain the prevalence of almost 100% dental fluorosis even in areas where the fluoride content of drinking water was below the permissible limit because the food items marketed and consumed need not strictly

be grown in the villages with low levels (1·4 mg litre^{-1}) of fluoride in water. Many other factors appear to contribute to the early and almost universal presence of dental fluorosis in schoolchildren (age group 6–18 years). As the study area constitutes one of the driest parts in South India with a mean maximum temperature of 36·4°C a higher than average water intake is necessitated. This suggests that reconsideration of the permissible levels in drinking water supplies is necessary to establish a more realistic basis. In this laboratory a more detailed investigation is being undertaken to establish more appropriate means for determining the recommended and maximum permissible levels of fluoride in drinking water. Other factors also contributed to widespread fluorosis, chief among them being (i) the liberal use of spices and condiments as a part of local dietary habits (Nanda 1974) during processing and cooking of the food for consumption added to the fluoride content; (ii) the lack of variety in the diet because of the generally prevailing illiteracy and poverty of the local population.

Defluoridation of drinking water supplies is indicated and methods suited to local conditions have been reported by the present authors (Venkateswara Rao and Mahajan 1988).

ACKNOWLEDGEMENTS

Grateful thanks are due to Sri Sathya Sai Institute of Higher Learning, Prasanthinilayam for funding the project (SR-IT 35) under which the work was carried out, and for providing all necessary facilities.

REFERENCES

Beddows C G, Kirk D 1981 Determination of fluoride ion in bovine milk using a fluoride ion selective electrode. *Analyst* **106** 1341–1344.

Cook H A 1970 Fluoride intake through tea in British children. *Fluoride* **3** 12–18.

Dabeka R W, McKenzie A D, Comachr H 1979 Microdiffusion and fluoride-specific electrode determination of fluoride in foods. *J Assoc Offic Anal Chem* **62** 1065–1069.

Gopalan C, Sastri B V R, Balasubramanian S C 1982 *Nutritive Value of Indian Foods*. National Institute of Nutrition, ICMR, Hyderabad, pp 29–32.

McClure F J 1949 Fluorine in foods. *Publ Hlth Rep (Wash)* **64** 1061–1074.

Nanda R S 1974 Fluoride content of North Indian foods. *Fluoride* 7(2) 114–116.

Venkateswara Rao K, Mahajan C L 1988 Defluoridation of drinking water in developing countries. Alternative and innovative technologies. In: *Hazardous and Industrial Wastes*, Proc 20th Mid-Atlantic Industrial Waste Conference, Howard University, Washington, DC, eds Varma M H & Johnson J H Jr. Hazardous Materials Control Research Institute, Washington, DC, pp 55–64.

Venkateswara Rao K, Mahajan C L 1989 Survey of water quality with special reference to fluoride content and endemic fluorosis in arid rural parts of South India (Anantapur District, Andhra Pradesh). *Asian Environ* **11** (2) (in press).

Villa A F 1970 Rapid method for determining fluoride in vegetation using an ion-selective electrode. *Analyst* **104** 545–551.

WHO 1970 *Fluorides and Human Health*. WHO Monograph Series No 59. WHO, Geneva, pp 32–40.

WHO 1984 *Guidelines for Drinking-water Quality, Vol 1, Recommendations*. WHO, Geneva.

J Sci Food Agric 1990, **51**, 281–284

Effect of Calcium on the Storage Life of Oro (*Antiaris africana*)

Olusegun O Lasekan*

Department of Food Science and Technology, University of Ibadan, Ibadan, Nigeria

(Received 15 March 1989; revised version received 28 June 1989; accepted 24 July 1989)

Key words: Oro (*Antiaris africana*), calcium concentration, chlorophyll, ascorbic acid, titratable acidity, texture, storage.

The effects of calcium on the ascorbic acid, chlorophyll, titratable acidity and texture (firmness) of stored oro (Antiaris africana (*Mill*)) *fruits (average weight 100 g) were studied. Three groups of the fruits were dipped in 20 g litre*$^{-1}$, *40 g litre*$^{-1}$ $CaCl_2$ *solutions and deionised water respectively for 10 min. The fourth group, which was untreated, served as the control and was also packaged in heat-sealed cellophane bags. The fruits were displaced and stored at ambient temperature (28* $\pm$ *2°C). The calcium-treated fruits which kept better were firmer and had a slight colour change (chlorophyll) which also produced a progressive increase in ascorbic acid level during storage. The titratable acidity of the fruits did not fluctuate as significantly as that of the untreated (control) fruits.*

Studies on fruit ripening (Tingwa and Young 1974; Suwwan and Poovaiah 1978) and on leaf senescence (Poovaiah and Leopold 1973; Ferguson 1984) have indicated that the rate of senescence often depends on the calcium status of the tissue and that, by increasing calcium levels, various parameters of senescence such as respiration (Faust and Shear 1972) and protein and chlorophyll content (Poovaiah and Leopold 1973) are altered. Changes in cell wall structure, membrane permeability and enzyme activation are known to influence various aspects of cell physiology, and it has been shown from recent experimental evidence that some of these cell functions are regulated in part by calcium and calmodulin (Poovaiah 1985).

This study is aimed at establishing the most effective calcium level necessary for extending the storage life of oro (*Antiaris africana* (Mill)).

The texture or hardness of the fruits was estimated using a Universal Instron Machine, model 1140 (Instron, High Wycombe, UK), with an 8-mm-dia plunger having a drive speed of 50 mm min^{-1}. The load cell force range used was 0–50 kg.

* Present address: Department of Food Science & Technology, Ondo State Polytechnic, PMB 1019, Owo, Ondo State, Nigeria.

J Sci Food Agric 0022-5142/90/$03.50

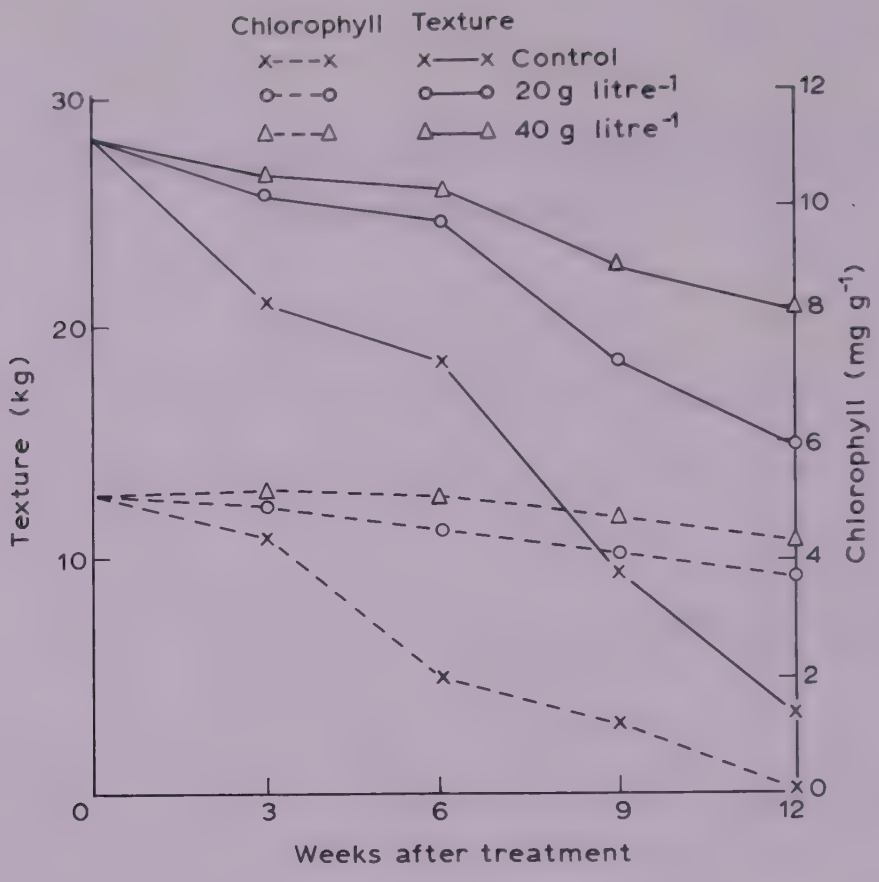


Fig 1.

For the estimation of chlorophyll, the fruits were peeled to about 0·5 mm thickness using a rotary knife. Two grams of fruit peel were finely ground with calcium carbonate and extracted in 80% acetone. Absorbance was measured at 663 and 645 nm using a model SP8-150 Pye-Unicam spectrophotometer. The ascorbic acid content was estimated using the dichlorophenol indophenol method (AOAC 1970) and titratable acidity was calculated as anhydrous citric acid by titration (Wardowski *et al* 1979).

The texture (firmness) of the untreated (control) fruits decreased gradually between weeks 1 and 6 but softened rapidly after week 6 (Fig 1). Within the same period the treated samples showed a gradual decrease and did not start softening until week 9. The rate of softening was slightly higher in fruits treated with 20 g litre^{-1} $CaCl_2$ solution between weeks 6 and 12 when compared with those treated with 40 g litre^{-1} $CaCl_2$. It was observed that fruits treated with calcium had higher bulk weight than the untreated (control) fruits; increasing the calcium level also increased the firmness of the fruits, an observation consistent with those of Conway and Sams (1984).

Immature oro fruits were dark green in colour with a chlorophyll content of 5·1 mg g^{-1} per fruit. The chlorophyll content decreased gradually with the exception of the control in which the decrease was progressively rapid (Fig 1). In the treated samples, a slight decrease was recorded between weeks 6 to 12 for the 40 g litre^{-1} $CaCl_2$ treated fruits whereas those treated with 20 g litre^{-1} started decreasing after week 3. It was also observed that the decrease in chlorophyll

TABLE 1
Effect of calcium on the titratable acidity (TA) and ascorbic acid (AA) content of *Antiaris africana*

Treatments	*Storage period (weeks)*									
	'O'		*3*		*6*		*9*		*12*	
	AA	*TA*	*AA*	*TA*	*AA*	*TA*	*AA*	*TA*	*AA*	*TA*
20 g litre^{-1} $CaCl_2$	158·0	3·1	179·2	3·6	189·0	3·2	197·6	2·8	195·0	2·9
40 g litre^{-1} $CaCl_2$	158·0	3·1	174·9	3·6	186·4	3·4	222·0	3·2	233·5	3·1
Water (deionised)	158·0	—	157·0	—	153·0	—	151·0	—	143·0	—
Control	158·0	3·1	155·0	3·4	151·0	2·6	141·5	1·1	149·0	2·2

AA, Concentration of ascorbic acid, mg kg^{-1} dry matter; TA, g anhydrous citric acid kg^{-1} dry matter.

content coincided with the ripening and softening of fruits. Thus, by the 12th week, fruits treated with 40 g litre^{-1} $CaCl_2$ were slightly green in colour whereas the second group of treated fruits were already showing a slight colour change. The inception of the decline in chlorophyll content was not clearly indicated by a distinct colour change. The untreated (control) fruits attained full colour change by week 6, and by the 12th week the fruits had darkened.

The effect of calcium on ascorbic acid content and titratable acidity of the fruit is shown in Table 1. The ascorbic acid content of the untreated (control) fruits decreased progressively. Those treated with deionised water followed a similar trend with a sharp decrease in week 12. There was a gradual increase in the ascorbic acid level of fruits treated with 20 g litre^{-1} $CaCl_2$. A similar trend was observed with fruits treated with 40 g litre^{-1} $CaCl_2$. This is probably an indication of calcium infiltration (Tingwa and Young 1974). The rate of increase in 40 g litre^{-1} $CaCl_2$ treated fruits was slow at the beginning, got progressively faster (being fastest between weeks 6 and 9, a period when the untreated (control) fruits and water-treated fruits suffered a significant drop in their respective ascorbic acid levels), and then slowed down after week 9. Although a slight decrease was observed in week 12, there was a significant increase in the ascorbic acid level when compared with those fruits treated with 20 g litre^{-1} calcium chloride during the same period. Thus, calcium not only played a special role in maintaining the cell structure (Paliyath 1984) but also altered the ascorbic acid content of oro.

The titratable acidity fluctuated significantly in untreated (control) fruits (Table 1). A pronounced decrease was observed between weeks 3 and 9 and an increase between weeks 9 and 12, a period during which all the untreated (control) fruits developed a strong 'fruity' aroma. The titratable acidity in fruits treated with 20 g litre^{-1} $CaCl_2$ decreased gradually between weeks 3 and 9, with a slight increase between weeks 9 and 12. Titratable acidity of fruits treated with 40 g litre^{-1} $CaCl_2$ decreased gradually between weeks 3 and 9 and slightly in week 12. The strong

'fruity' aroma developed in the control between weeks 9 and 12 was absent in both treated samples during the same period.

It was observed that calcium plays a significant role in influencing some parameters of senescence in oro. Of the two calcium treatments used, the most effective treatment was that of 40 g litre^{-1} calcium chloride concentration which produced firmer fruits of higher ascorbic acid level. Although there was fluctuation in the titratable acidity of calcium-treated fruits, this was not as significant as that of the untreated (control) fruits. By the 9th week of storage, the untreated (control) fruits had already attained senescence whereas the calcium-treated fruits were yet to attain senescence even by the 12th week. This is an indication of the preservative action of calcium.

REFERENCES

AOAC 1970 *Official Methods of Analysis* (11th edn), sec. 39.051. Association of Official Analytical Chemists, Washington, DC.

Conway W S, Sams C E 1984 Possible mechanisms by which postharvest calcium treatment reduces decay in apples. *Phytopathology* **24** 208–210.

Faust M, Shear C E 1972 The effect of calcium on respiration of apples. *J Amer Soc Hort Sci* **97** 437–440.

Ferguson I B 1984 Calcium in plant senescence and fruit ripening. *Plant Cell Environ* **7** 447–448.

Paliyath G 1984 Membrane fluidity in senescing apples: effects of temperature and calcium. *Plant Cell Physiol* **25** 1083–1086.

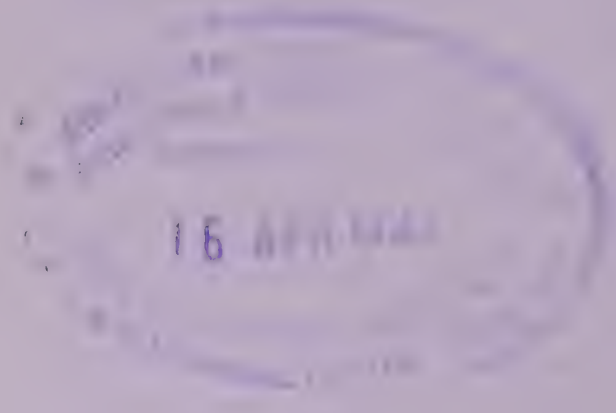
Poovaiah B W 1985 Role of calcium and calmodulin in plant growth and development. *Hort Sci* **20** 347–349.

Poovaiah B W, Leopold A C 1973 Deferral of leaf senescence with calcium. *Plant Physiol* **52** 236–239.

Suwwan M, Poovaiah B W 1978 Association between elemental content and fruit ripening in rin and normal tomatoes. *Plant Physiol* **61** 883–886.

Tingwa P O, Young R E 1974 The effect of calcium on the ripening of avocado (*Persea americana* Mill) fruits. *J Amer Soc Hort Sci* **99** 540–543.

Wardowski W, Soule J, Grierson W, Westbrook G 1979 Florida citrus quality tests. *Bull 188*. Florida Cooperative Extension Service, Institute of Food and Agricultural Science, Gainesville, FL.



RANCIDITY IN FOODS

Second Edition

edited by **J.C. Allen,** *Dean, Research and Innovation, The North East Wales Institute, Deeside, Clwyd, Wales, UK and* **R.J. Hamilton,** *Principal Lecturer, Division of Chemistry, School of Natural Sciences, Liverpool Polytechnic, Liverpool, UK.*

22 x 14 cm xii + 234 pages 47 illus. £37.00/US$66.50 ISBN 1- 85166-327-4 1989

Since the first edition, there has been a rapidly increasing concern about the safety of food by the general public. Pressure from consumer groups, an increasing volume of food legislation (particularly from the European Community) and public concern for 'healthy eating' is placing an increased burden on food manufacturers to ensure a high product standard. The responsibility often rests on the food technologist, who must be aware that quality is best achieved by knowledge of his raw materials and process, rather than by inspection and rejection of finished product.

'Rancidity in Foods' aims to provide the food technologist with the basic facts and industrial relevance about the fundamental problem in food chemistry. It adopts the method used in the successful first edition, of presenting authoritative chapters on each relevant area, written by acknowledged experts in their field.

As before, the emphasis is on practical application. However, the book has been revised to include new developments, and enlarged to include new chapters on the use of bulk quantities of oils and fats, and on rancidity in meat products.

ELSEVIER SCIENCE PUBLISHERS
Crown House, Linton Road, Barking, Essex IG11 8JU, UK.

In USA and Canada:
ELSEVIER SCIENCE PUBLISHERS CO. INC.,
P.O. Box 882, Madison Square Station, New York, NY 10159
Note: in NY state, please add applicable tax

FIRST ANNOUNCEMENT

Bioflavour '90

Forum Hotel, Glasgow, Scotland
26th–28th September 1990

Bioflavour '90 is designed to bring together renowned speakers from around the world to present recent developments in the origin, assessment and application of biologically produced flavours. The conference will be of interest to all those in the food and beverage, flavour and fragrance industries worldwide who require an up-to-date understanding of flavours, aromas and fragrances and their generation and use, in the wider context of industry rather than the scientific and technical literature.

The conference will last for three days, and is divided into five major themes:

- Assessment of flavour
- Use of computers in flavour assessment
- Flavours arising from breeding
- Biotechnology
- Flavours arising from fermentation and processing

A poster session has been scheduled to permit active researchers to present their latest results. The proceedings of the conference will be published by Elsevier Science Publishers.

TITLES AND SPEAKERS WILL INCLUDE:

Predicting acceptability from flavour data
Prof Howard Schutz, University of California, Davis, USA

Sensory analysis
Dr Peter Durr, Swiss Federal Research Station for Fruit-Growing, Viticulture & Horticulture, Wadenswil, Switzerland

Food acceptability
Dr Anthony Williams, Sensory Research Laboratories Ltd, Bristol, England

Psychology of flavour
Dr Steve van Toller, University of Warwick, England

Overview: Data collection and statistical analysis
Dr J R Piggott, University of Strathclyde, Scotland

Matching sensory with instrumental analysis
Dr Magni Martens, University of Oslo, Norway

Product optimisation
Prof Mario Bertuccioli, University degli Studi della Tuscia, Viteroo, Italy

Effects of breed and variety on flavour
Dr A Paterson, University of Strathclyde, Scotland

Meat flavours
Dr Don Mottram, University of Reading, England

Natural flavours for alcoholic beverages
Dr Jacques Bricout, Centre de Recherche Pernod Ricard, France

Wine Flavour
Prof Ann Noble, University of California, Davis, USA

Distilled beverage flavour
Dr H Maarse, CIVO-Instituten TNO, The Nethe

Cocoa flavour
Dr B D Baignie, Cadbury Schweppes, Reading, England

ABSTRACTS

Authors are requested to submit two copies of abstracts in English, typed in double space, not exceeding one page of A4 including title, name author(s) and affiliation. Both copies should be to:

Dr J R Piggott
University of Strathclyde
Department of Bioscience and Biotechnol
James P Todd Building
131 Albion Street
Glasgow G1 1SD
Scotland

The deadline for submission of abstracts is 30t 1990. An acknowledgement of receipt will be you.

Bioflavour '90

Please send me further details and registration information

- ☐ I am interested in participating as a delegate
- ☐ I am interested in submitting an abstract
- ☐ I am interested in having a Poster Panel

Title of Poster _____

(Please tick as many boxes as are relevant)

SURNAME _____

TITLE _____ INITIALS _____

COMPANY/INSTITUTE _____

_____

ADDRESS _____

_____

_____

_____

POST CODE _____ COUNTRY _____

Please return to: Penny Moon
Conference Manager
Elsevier Science Publishers
Mayfield House
256 Banbury Road
Oxford OX2 7DH, UK
Tel: (0865) 512242
Fax: (0865) 310981

ABBREVIATED INSTRUCTIONS TO AUTHORS

(see first issue each year for full version)

1. Papers and correspondence to
The Managing Editor, *Journal of the Science of Food and Agriculture*, Society of Chemical Industry, 14/15 Belgrave Square, London SW1X 8PS, UK. Telephone: (01) 235 3681. Telefax: (01)823 1698.
Papers from North America may be sent to
Dr D S Reid, Department of Food Science & Technology, University of California, Davis, CA 95616, USA.
or to
Prof P J Van Soest, Department of Animal Science, New York State, College of Agriculture and Life Science, Cornell University, Frank B Morrison Hall, Ithaca, NY 14853, USA.

Authors will receive an immediate acknowledgement of receipt of their paper followed, within 3 months by notification of acceptance or rejection. Authors should refer to the **detailed** instructions available from the Managing Editor and in first issue each year.

2. Manuscripts may be either a formal **full length** paper or a **shorter research communication**. The latter must not exceed 1250 words and may include up two figures or tables, or one of each; the first paragraph should function as an abstract, experimental detail should be minimal but unambiguous and may be included in the legends to the figures or tables; sectionalisation is inappropriate and no acknowledgements should be given except against specific items in the text; keep citations to a minimum.

3. Type all manusccipts in double spacing on one side of A4 paper leaving adequate left and right margins. Submission will be taken to signify that the manuscript is not being simultaneously considered for publication elsewhere. The Editors accept papers only in English.

4. The title should be concise, specific and explain the nature of the paper. Where it exceeds 60 characters suggest a running title. Each author's name must include the customary forename in full and other initials (eg Arthur B Smith). Give the full address where the work was done and that for correspondence and proofs.

5. Provide an Abstract (150–200 words) reporting concisely the purpose and results of the work and keywords for the main copies of the paper including any in the title

6. The **SI system** should be used for all data. The full stop should not be included in abbreviations, eg m (not m.); Use the form g kg^{-1}, avoid g 100^{-1}, and % for reagent concentrations. Unusual symbols (including Greek lettering) should be defined in the left margin at the first mention. Abbreviations of chemical or other names should be defined when first mentioned unless commonly used and internationally known and accepted.

7. Number tables consecutively and supply each on a separate sheet. Include figures and photographs only if essential and number then in a single sequence. Use only Arabic numerals. Line diagrams should be drawn in black ink or supplied as a glossy print and should require the same degree of reduction. Insert numerals, points and essential lettering clearly and in proportion to the detail. Handwritten or typed lettering is **not** acceptable nor are computer-drawn diagrams unless prepared on a high quality printer. Photographs should be submitted as clear black and white prints on glossy paper (see the detailed instrutions for guidance when illustrations of electrophoresis patterns are essential). Write the number of each illustration, together with the title and author of the paper, on the reverse side using a soft pencil.

8. Use roman numerals (I, II, etc) to number each chemical structure, preparing these as illustrations as described above.

9. Carry out **and describe** appropriate statistical analyses. Give the form of errors and their statistical significance. Further guidance is given in the *Journal of the Science of Food and Agriculture* Vol 42 Number 1 and further copies are available from the Managing Editor.

10. References to published work should be indicated using author's name and date at the appropriate place in the text (Green 1984a; White and Jones 1986) and a list of references in alphabetical order should appear at the end of the paper. All references in this list should be indicated at some point in the text and vice versa. Refer to unpublished work entirely in the text thus: (Smith A B, unpublished), (Brown C D, pers comm). Abbreviate journal titles as in Chemical Abstracts or Biological Abstracts. When quoting patents give the name of the applicant, the year of publication, the title, the country and patent number.

11. Author's proofs should be returned without delay. No new material may be inserted in the text at proof stage. Papers will normally be published in order of acceptance by the Editors, although this may occasionally be varied.

12. **THERE ARE NO PAGE CHARGES**. Twenty-five offprints of each article are supplied free of charge. Additional offprints can be ordered at current printing prices.

SUBSCRIPTIONS

1990—Four volumes, four issues per volume. Subscription price (Volumes 50–53); UK delivery, £225.00; Overseas delivery, £247.00/$420.00. The £ sterling price is definitive; US$ prices are subject to exchange rate fluctuation. All prices include postage and packing. All journals are distributed worldwide by air-speeded delivery at no extra cost to the subscriber. Subscription orders should be addressed to

ELSEVIER SCIENCE PUBLISHERS LTD
Crown House, Linton Road, Barking, Essex IG11 8JU, England

in the United States and Canada: For further information contact

Elsevier Science Publishing Co., Inc., Journal Information Center
655 Avenue of the Americas, New York, NY 10010. Tel. (212) 989-5800

JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE VOL. 51 NO. 2 1990

(Abstracted/indexed in: *AGRICOLA database, BIOSIS (Biological Abstracts); Biological and Agricultural Index; CAB Abstracts; Chemical Abstracts; Excerpta Medica/Embase; Index Medicus; Current Contents; Science Citation Index*)

CONTENTS

SCI Published for the SOCIETY OF CHEMICAL INDUSTRY by ELSEVIER APPLIED SCIENCE